INFLUENCE OF AUTOINDUCER 2 (AI-2) AND AI-2-LIKE INHIBITORS GENERATED FROM GROUND BEEF ON *ESCHERICHIA COLI* O157:H7 PROTEIN EXPRESSION

A Dissertation

by

KAMLESHKUMAR ARVINDKUMAR SONI

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2008

Major Subject: Food Science and Technology

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ABSTRACT

Influence of Autoinducer 2 (AI-2) and AI-2-like Inhibitors Generated from Ground Beef on *Escherichia coli* O157:H7 Protein Expression. (May 2008)

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Autoinducer 2 (AI-2) molecules produced by bacterial cells are thought to be involved in controlling a variety of bacterial cellular processes by coordinated gene and protein expression. Previous work in our laboratory has shown that ground beef contains compounds that can interfere with AI-2-mediated bioluminescence expression in *Vibrio. harveyi*. The underlying hypothesis of this work was that AI-2 molecules affect the protein expression in *Escherichia coli* O157:H7 and AI-2 inhibitory molecules negate the influence of AI-2 molecules. The main objectives of this study were to identify, characterize, and isolate the factors responsible for inhibition of AI-2 molecules from ground beef extracts, elucidate the role of LuxS/AI-2 cell signaling system in *E. coli* O157:H7 protein expression, and determine if inhibitory factors present in ground beef extract can negate the influence of AI-2 molecules on the protein expression. Using a solvent extraction procedure and gas chromatography analysis, AI-2 inhibitory factors present in ground beef extracts were identified as both medium and long chain fatty acids. When identified fatty acids were tested at different concentrations for AI-2

inhibition, AI-2 inhibition ranging from 25% to 90% was observed. Both ground beef extracts and mixture of selected fatty acids also resulted in 2- to 4-fold reduced AI-2 influenced biofilm formation by *E. coli* K12 cells. Identification of LuxS/AI-2-mediated protein expression in *E. coli* O157:H7 was conducted using two dimensional gel electrophoresis. Protein expression analysis showed that the LuxS/AI-2 system modulates the expression of proteins involved in different cellular processes such as carbohydrate and amino acid metabolism, stress response, and formation of flagella and motility. When AI-2 inhibitory factors were added along with AI-2 molecules, the expression patterns of three AI-2-influenced proteins (GlmS, SpeE, and NikA) were changed suggesting that AI-2 inhibitors can negate the influence of AI-2 molecules on protein expression of selected proteins. Collectively, these results highlight that proteins associated with different cellular processes in *E. coli* O157:H7 can be modulated depending on whether cells are in contact with AI-2 molecules in the presence or absence of AI-2 inhibitory factors.

DEDICATION

My doctor of philosophy and this dissertation are dedicated to my wife, parents and grand parents, uncle and aunty, and sister.

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CHAPTER I

INTRODUCTION

RATIONALE

Results from previous studies have shown that a variety of bacterial cell processes such as growth, sporulation, toxin production, virulence, antibiotic synthesis, and motility in bacterial cells are coordinately regulated at the gene expression level by a variety of intra- and inter-cellular *autoinducer* molecules in a process termed quorum sensing (QS) (Bassler *et al.*, 1997; Surette and Bassler, 1998; Taga and Bassler, 2003). Different autoinducer (AI) molecules including AI-1, AI-2, AI-3, oligopeptides, indole, cyclic peptides, and the *Pseudomonas* Quinolone Signal have been previously described in the literature (Bansal *et al.*, 2007; Miller *et al.*, 2004; Sperandio *et al.*, 2003; Taga and Bassler, 2003). The autoinducer 1 (AI-1) is thought to be highly species-specific (Cao *et al.*, 2001), while autoinducer 2 (AI-2) is thought to serve as a 'universal' bacterial signal for inter-species communication (Surette and Bassler, 1998). Autoinducer 3 (AI-3) is thought to be involved in communication between the bacterial cells and the host's signaling system, whereas AI-peptides are used by Gram-positive bacteria (Bassler, 2002; Sperandio *et al.*, 2003). Among the different AI molecules, AI-2 has been considered the universal signaling molecule, since the *luxS* gene, which is involved in

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This dissertation follows the style of *Molecular Microbiology*.

the production of AI-2, is widely conserved among the different bacterial species (Xavier and Bassler, 2003). In addition, catecholamines such as epinephrine (epi) and non-epinephrine (NE) produced by human epithelial cells mimic the action of the bacterial QS molecule AI-3, suggesting a possible role of autoinducer molecules in bacterial-host interactions (Sperandio *et al.*, 2003).

New information continues to be obtained about the bacterial species that possess QS systems, the levels of auto-inducer molecules within the host, the genes that are controlled by QS, and the identity of the signaling molecules. It has been demonstrated that bacterial cells can produce autoinducer molecules when present in food matrices (Bruhn *et al.*, 2004; Cloak *et al.*, 2002); however, very little is known about how cell signaling influences spoilage and pathogenic bacteria in foods in the context of food safety and food processing (Pillai and Jesudhasan, 2007; Smith *et al.*, 2004). Previously, it was shown that some food matrices possess a characteristic ability to obstruct these autoinducer signaling molecules (Choo *et al.*, 2006; Lu *et al.*, 2004; Vattem *et al.*, 2007). Identification and characterization of the prevalent compounds responsible for the inhibition of autoinducer molecules may have value in controlling AI-2-influenced traits of particular pathogenic organisms.

Escherichia coli O157:H7 is a widespread infectious human pathogen responsible for gastrointestinal illnesses, including hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC). Like many other Gram-negative bacterial species, *E. coli* can also produce AI-2-like molecules. Even though AI-2 molecules are known to regulate gene expression, currently, there is no report correlating the presence of in vitro-

synthesized AI-2 molecules with protein expression in *E. coli* using high throughput techniques such as two dimensional gel electrophoresis.

Overall focuses of our studies were to determine the influence of AI-2 molecules on protein expression of *E. coli* O157:H7, and to determine if the AI-2 inhibitory factors derived from ground beef would negate the influence of AI-2 molecules on protein expression. The underlying hypothesis of this work was that AI-2 molecules affect protein expression in *E. coli* O157:H7 and AI-2 inhibitory factors in beef negate the influence of AI-2 molecules.

OBJECTIVES

1) Identify, characterize, and isolate the factors responsible for inhibition of AI-2 molecules from ground beef extracts and determine the ability of isolated compounds to interfere with the AI-2 response using a *Vibrio harveyi* reporter strain based AI-2 bioassay.
2) To elucidate the role of the Lux/AI-2 cell signaling system in *E. coli* O157:H7 by monitoring cellular protein expression using two dimensional gel electrophoresis.
3) Determine if inhibitory factors present in ground beef extract can interfere with the AI-2 response in *E. coli* O157:H7, and thereby negate the influence of AI-2 molecules on the protein expression.

CHAPTER II

LITERATURE REVIEW

FOODBORNE DISEASES IN THE UNITED STATES

Foodborne disease has been defined by World Health Organization as "any disease of an infectious or toxic nature caused by, or thought to be caused by, the consumption of food or water". Food commodities that consist or externally contaminated by chemical or microbial agents which are harmful to human body possess a significant food safety risk. Examples of causative chemical agents in food include toxic alkaloids present in green or sprout potatoes, anti-nutritional factors such as phytate and trypsin inhibitors present in plant legume and foods contaminated by toxic compounds such as aflatoxins (Carbonaro *et al.*, 2001; Robens and Richard, 1992; Wang *et al.*, 2005c). The causative microbial agents of foodborne illness include bacteria, viruses, and parasites. Comparing the food hazards caused by chemicals and microbial agents, it has been established that the microbial hazard is of paramount importance (McCabe-Sellers and Beattie, 2004). In most instances, foodborne illnesses caused by microbial agents are generally mild, limited to gastroenteritis, and not life threatening to adults. However, immunocompromised subjects such as elderly, young, and pregnant women can be at high risk of foodborne illnesses (Kendall *et al.*, 2003). In order for microbial agents to cause illness, sufficient numbers of cells need to be consumed.

Foodborne diseases caused by microbial agents are of substantial health and economic burden in the United States and across the world. It is estimated that

foodborne diseases cause about 76 million illnesses, 325,000 hospitalizations, and approximately 5,000 deaths each year in the United States (Mead *et al.*, 1999). Among total number of foodborne diseases, 38.6 million illnesses are caused by the known agents including 5.2 million due to bacteria, 2.5 million due to parasites, and 30.9 million due to viruses (Mead *et al.*, 1999). It is estimated that foodborne diseases cost about seven billion dollars to the economy of the United States. The economic losses that occur due to foodborne diseases include, health care cost, loss of income by the affected individuals, loss of productivity, costs of investigation of an outbreak, and loss of income due to loss of sales (Frenzen *et al.*, 2005).

The source of microorganisms in food varies depending on types of food. In case of meat products, the microflora present on the surface (hide, hair, horns) and in the gastrointestinal tract are the primary sources of contamination (Small *et al.*, 2006). Cross contamination from the equipment, utensils, workers, air, water, and packaging material can also contribute to the presence of microorganisms. In case of fruits and vegetables, soil, water, animals, fertilizer, dust particle, packaging equipment, vehicles, and humans are some of the prime sources of contamination (Heaton and Jones, 2007). Due to the fact that fruits and vegetables are often consumed with minimal preparation, they possess significant food safety risk if contaminated. Water activity, relative humidity, pH, nutrient content, antimicrobial constituents, temperature, and time of storage are some of the other key factors that dictate type of microorganism that can survive or grow in particular food matrices (Gock *et al.*, 2003; Himathongkham *et al.*, 1999). For instance, most of the Gram-negative and Gram-positive microflora needs water activity

of more than 0.9, whereas fungi such as *Xeromyces bisporus* can grow in food matrices with water activity as low as 0.6 (Gock *et al.*, 2003). If food commodities with low water content are stored in a high humidity environment, water from the gas phase will transfer to the food and increase its water content. Microbial growth on foods also depends on the nutrient content of food matrices. For example, the dominant microflora on cereals are amylolytic bacteria that can produce starch-degrading enzymes. Most of the bacteria show optimum growth in pH range of 6 to 8; however, a group of lactobacilli and acetic acids bacteria can grow optimally between pH of 5 to 6. Antimicrobial constituents of food such as lactoferrin, lactoperoxidase, and lysozyme can limit or prevent the microbial growth (Shahani and Chandan, 1979). Temperature is another important factor that determines the nature and type of microorganism that can be present on a particular food. For instance, food products stored at low temperatures are likely to contain psychrophiles such as *Pseudomonas* and *Listeria monocytogenes.*

Bacterial pathogens. *Salmonella*, *Listeria monocytogenes*, *Campylobacter jejuni, E. coli* O157:H7 and *Yersinia enterocolitica* are the major contributors to foodborne illnesses. It is estimated that among these prevalent foodborne pathogens, *Campylobacter* causes 2 to 4 million cases, *Salmonella* causes 1.4 million cases, and *E. coli* O157:H7 causes 73,000 cases of illness each year (Mead *et al.*, 1999). Consumption of egg and poultry products has been identified as a major vehicle for *Salmonella* infections. Most *Salmonella* serotypes are considered as human pathogens and, therefore, they are an important causative agents of human illnesses world wide (Foley and Lynne, 2007). *Salmonella* serotypes are present in a variety of food products (such

as milk, meat, poultry, and eggs), since they are natural inhabitants of gastrointestinal tracts of food animals, wild animals, bird, and pets. The symptoms of *Salmonella* infection include mild fever, nausea, vomiting, abdominal pain, and diarrhea. Consumption of chicken products prepared at commercial establishments is often a high risk factor for *Campylobacter*-associated food infections (Havelaar *et al.*, 2007). Symptoms of *C. jejuni* infection are diarrhea, fever, abdominal pain, nausea, headache and muscle pain and these symptoms are often self limiting. *Listeria monocytogenes* is the only important human pathogen within genus *Listeria.* The prevalence of *Listeria monocytogenes* is mainly due to its wide-spread distribution, and ability to grow on non-acid foods even at low temperatures (Cossart, 2007). Because of its wide-spread distribution, *Listeria* is often isolated from wide range of food products such as raw milk, soft-ripened varieties of cheese, ice cream, raw vegetables, poultry, and raw meats (Cossart, 2007). The infective dose of *Listeria monocytogenes* is greater than 100 CFU and the disease conditions caused by *Listeria monocytogenes* is called "listeriosis". Listeriosis results in symptoms such as flu-like illness to meningitis (Lennon *et al.*, 1984). Besides the direct bacterial infection, toxins produced by several microorganisms can also cause foodborne-related illnesses. For instance, botulism toxin produced by *Clostridium botulism* can cause slurred speech, difficulty in swallowing, weakness in muscle and sometime muscle paralysis (Abgueguen *et al.*, 2003).

A national health objective of reducing the major bacterial foodborne illnesses by the end of 2010 was established by the Department of Health and Human Services in 2000. As a result of these efforts, the Centers for Disease Control and Prevention (CDC)

surveillance data for 2006 revealed that the incidence of infections caused by *Campylobacter*, and *Listeria* are on the decline. However, no decrease in the incidence of infections caused by *Escherichia coli* O157: H7 and *Salmonella* was observed (Morbidity and Mortality Weekly Report, 2006; 2007). Effective pathogen intervention strategies are critically important to combat foodborne diseases. Efforts to reduce the rate of foodborne illness associated with high-risk food safety pathogens should be targeted to high-risk foods pro-actively, rather than attempting to treat infected persons.

Viral pathogens. Among different groups of viruses, norovirus and Hepatitis A virus are major contributors to foodborne outbreaks (Atreya, 2004). Viruses are obligate intracellular parasites; therefore, they require particular host cells for replication. The multiplication of viruses does not occur in foods, therefore, food is considered as a passive vehicle in transmission and infection. The typical syndrome caused by viral-associated food infections are gastroenteritis, however; viruses to other body organs such as liver can cause life threatening illnesses (Katz *et al.*, 1996). Use of human excrement as a fertilizer can introduce enteric viruses to fresh produce such as vegetables and salads (Nakagawa *et al.*, 2006). The contamination of food with viruses can occur anywhere from the farm to the distribution system; however, infected personnel who handle the foods are the leading cause for the viral infections being transmitted through food (Nakagawa *et al.*, 2006). In 1982, a large outbreak of viral infection involving over 3,000 people occurred due to unhygienic conditions (improper washed hands after toilet) created by a bakery chef. Shellfish are also often implicated to harbor enteric viruses (Shieh *et al.*, 2003). Shellfish are often grown in shallow waters, which are often

contaminated with sewage. The practice of feeding shellfish extracted organic matter by filtering sea water also results in concentration of viruses and bacteria (Mesnage *et al.*, 2007). In Shanghai, almost 300,000 people were reported ill in 1998 due to consumption of hepatitis A contaminated shellfish (Cooksley, 2000). Currently available techniques for the virus detection include observation of plaque in a cell monolayer, immunoassay-based approach, and polymerase chain reaction with nucleic acid probes (Brown, 2006). Due to the complex nature of viral detection methods, testing of foods for the presence of viruses is more limited then testing for the presence of bacterial pathogens (Brown, 2006). Further, most of our current strategies to limit foodborne illness are mainly targeted to bacterial pathogens and these strategies are effective against viruses only to a limited extent.

Parasites as pathogen. Parasites are also responsible for causing an enormous food safety risk (Schnieder, 2003). Parasites are eukaryotic organisms and vary in size and complexity from unicellular to multi cellular organisms. *Toxoplasma, Cryptosporidium,* and *Giardia* are some of the key emerging parasites, and they often result in symptoms such as flu-like illness, fatigue, and muscle pain (Yeh *et al.*, 2001). Among the total foodborne related illnesses, *Toxoplasma* is responsible for approximately 21% of the illnesses and the resultant disease is known as toxoplasmosis (Mead *et al.*, 1999). The definitive host for this pathogen is the domestic cat but vertebrate animals including humans can be infected by oocysts (Webster, 2007). Plant eating animals can become infected by eating grass contaminated with cat feces and subsequently serve as its transmission route (Volkman and Hartl, 2003). *Toxoplasma* in an infected person

remains in the latent state, however, infection of immunocompromised individuals can be life threatening. *Cryptosporidium* is mainly associated with waterborne outbreaks and in the last ten years at least 19 outbreaks have occurred (Fayer, 2004). The major transmission route for *Cryptosporidium* is the environmentally robust oocyst secreted by an infected individual (Fayer *et al.*, 2000; Fayer, 2004). Direct contamination of food with *Cryptosporidium* is very uncommon, but the use of contaminated water in food processing or by farm animals can be responsible for its presence in food. Like *Cryptosporidium, Giardia* is also associated with water and person-to-person transmission due to poor hygiene (Brandonisio *et al.*, 2000). *Giardia* cysts have been isolated from vegetable salads and fruits and they may be transmitted whenever foods are washed with contaminated water or handled by an infected individual (Smith *et al.*, 2007). *Giardia* cyst are resistant to water chlorination process, but cooking temperature is enough for their inactivation (Jarroll *et al.*, 1981).

ESCHERICHIA COLI O157:H7 AS A FOODBORNE PATHOGEN

Background of *Escherichia coli* O157:H7. *Escherichia* is a genus of Enterobacteriaceae family and *E. coli* is the type species of this genus. It is a catalase positive, oxidase negative, Gram-negative, short non-spore forming rod shaped bacterium. Based on biochemical differentiations, most strains of *E. coli* are indole- and methyl red- positive and Voges Proskauer- and citrate- negative. *E. coli* is a mesophile in nature and grows between 7ºC (minimum) and 50ºC (maximum) with an optimum temperature of 37ºC. It is a facultative anaerobe in nature and commensal microflora of

the warm-blooded animals. Even though most *E. coli* strains are generally harmless, it can be an opportunistic pathogen and can cause a wide range of infections including urinary tract infections, hemolytic uremic syndrome (HUS), hemorrhagic colitis (HC), and gastrointestinal illnesses (Moxley, 2004).

The pathogenic strains of *E. coli* have been classified in six groups based on their virulence properties (Han and Lee, 2006; Moxley, 2004). These pathogenic groups consist: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), diffusely adherent *E. coli* (DAEC), and enteroaggregative *E. coli* (EAEC). Among these, only the first four groups are of concern in foods and water (Kaper, 1998). These four groups are capable of causing diarrheal disease, but in the case of EHEC, the diarrheal disease can progress to potentially fatal hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC). Among the different serotypes of EHEC, *E. coli* O157:H7 occurs more frequently is associated with foodborne illnesses worldwide (Kaper, 1998; Seto *et al.*, 2007). It has been reported that as few as 100 cells of *E. coli* O157:H7 are enough to cause infection (Teunis *et al.*, 2004). The distinct characteristic of *E. coli* O157:H7 from other *E. coli* is that it cannot ferment sorbitol in MacConkey-sorbitol agar and lack B- glucuronidase activity (Orth *et al.*, 2007). They can also be differentiated based on the production of shiga-toxin and characteristic cytotoxic effect on HeLa cells (Sperandio *et al.*, 2003).

***Escherichia coli* O157:H7 pathogenicity and infection process.** *E.* coli O157:H7 is an extracellular bacterial pathogen and a well-studied serotype of enterohaemorrhagic *Escherichia coli* strains. Interaction between *E.* coli O157:H7 and host cells has been

viewed as a three stage process. In the first step, expression of different surface factors such as fimbriae, outer membrane proteins and chaperone by *E.* coli O157:H7 causes initial interaction with the host epithelial cells (Spears *et al.*, 2006). The second stage of *E.* coli O157:H7-mediated pathogenicity involves type three secretion systems (TTSS) that inject secreted proteins from the bacterial cytoplasm directly into host cells. TTSS is required for intimate attachment of the bacteria, which results in a characteristic attaching and effacing (AE) lesion on the intestinal mucosa. TTTS apparatus is encoded by the Locus of Enterocyte Effacement (LEE) pathogenicity island important for host colonization. The LEE region is divided into five major polycistronic operons named LEE1 through LEE4 and Tir (translocated intimin receptor) (Sperandio *et al.*, 1999). When present on the surface of host cells, bacterial cells secrete and transport bacterial effector proteins into the host cells. TTSS is thought to generate a pore in the host cells permitting the translocation of secreted protein (Vallance and Finlay, 2000). In the third stage, translocated proteins activate signaling pathways within the host cells causing the reorganization of the host actin cytoskeleton and intimate attachment of bacterial cells (Spears *et al.*, 2006). The outer membrane bacterial protein, intimin, mediates intimate attachment to host cells by binding to the 90-kDa translocated bacterial receptor protein, Tir. The reorganization of actin forms a pedestal-like structure on which adherent bacteria reside. *Escherichia coli* O157:H7 leads the subversion of host signaling pathways to aid in infection once the intimate attachment and pedestal-like structure has occurred. The host cellular changes include increased inositol phosphate and intracellular calcium, activation of protein kinase, and induction of inflammatory

response (Kolodkin-Gal *et al.*, 2007). The outcome of diarrhea is thought to be the result of dramatic loss of absorptive microvilli due to AE lesion, reduced integrity of epithelial monolayer (Philpott *et al.*, 1996), disturbance in normal host-prokaryote equilibrium (Vallance and Finlay, 2000), and increased cellular permeability caused by the recruitment of neurophils and polymorphonuclear leucocytes at the site of infection (Ulshen and Rollo, 1980).

Acquisition of bacteriophage encoding shiga-like toxin (Stxs) by *E.* coli O157:H7 is one of the major differentiating factors compared to other diarrheagenic *E. coli* strains (Spears *et al.*, 2006). Different variants of Stxs have been reported of which Stx1 and Stx2 are predominant. Binding of glycolipid receptor GB3/CD77 in host cells with B-subunits of Stx1 or Stx2 causes inhibition of protein synthesis. In humans, GB3/CD77 receptors present on glomerular cells of the kidney result in serious damage to this organ and serious outcome of infection. In cattle, GB3/CD77-positive cells are present on intestinal crypts instead of kidney glomerular cells (Hoey *et al.*, 2002). Therefore, while both human and cattle are susceptible to *E.* coli O157:H7 colonization, the difference in GB3/CD77 receptor distribution is a key aspect in the outcome of disease (Spears *et al.*, 2006). Apart from TTSS and Stxs, serine proteases exported by auto-transporter mechanisms seem to play a role in pathogenicity, but the precise targets for these proteases are unclear (Henderson *et al.*, 2004).

Association of *E. coli* O157:H7 with foodborne outbreaks. Food animals are the most important source of human infections related to *E. coli* O157:H7. Diseased and healthy ruminants, especially cattle, are the main reservoirs of this pathogen (Li and Hovde,

2007; Todd and Dundas, 2001). Transfer of *E. coli* O157:H7 to meat products usually occurs during the slaughtering process where removal of the hide or gastrointestinal tract contaminates the carcasses (Rangel *et al.*, 2005). In addition, presence of this microorganism on the udder of cow or milking instruments may lead to its transfer to milk and milk products (Rangel *et al.*, 2005). Even though contaminated meat and milk from infected animals is a major transmission route for *E. coli* O157:H7, use of contaminated manure and irrigation water on agricultural field crops can be responsible for its transfer to fruits and vegetable produce (Mead and Griffin, 1998; Todd and Dundas, 2001). Apart from food and water, the infected individual can serve as a transmission route of this pathogen (Afza *et al.*, 2006). Bacteria from the feces of infected individuals can spread to others if proper hygienic conditions are not maintained. Family members of toddlers can be at high risk, since toddlers are not toilet trained. Use of recreational facilities by an infected individuals or mixing of potable water mains with sewage system can serve as transmission routes for this pathogenic microorganism (Verma *et al.*, 2007).

Escherichia coli O157:H7 was first identified as a foodborne pathogen in 1982 when an outbreak of hemorrhagic colitis (HC) occurred with the consumption of hamburger from fast food restaurants (Rangel *et al.*, 2005). Since then, there has been a number of foodborne outbreaks associated with *E. coli* O157:H7. Information about the total number of outbreaks occurring between 1982 and 2002 were summarized by Rangel et al (Rangel *et al.*, 2005). During this period, 350 outbreaks were reported in 49 states, representing 8,598 cases, 1,493 hospitalizations, 354 cases of hemolytic uremic

syndrome, and 40 deaths. Hussein and Bollinger evaluated reports published in the past three decades concerning the prevalence of *E. coli* O157:H7 in beef products (Hussein and Bollinger, 2005). The results showed prevalence rates of 0.1 to 54.2% in ground beef, 0.01 to 43% in carcasses, 1.1 to 36.0% in retailed cuts, and 0.1 to 4.4% in sausages. Outbreak-associated cases of *E. coli* O157:H7 infection accounted for at least 36 in 2004, 473 cases in 2005, and 88 cases in 2006 another (Morbidity and Mortality Weekly Report, 2006). In 2005, 71 cases of HUS occurred in children aged less than 18 years; of which 47 cases occurred in children aged less than 5 years (Morbidity and Mortality Weekly Report, 2007). In 2006, three large multistate outbreaks of *E. coli* O157:H7 infections associated with produce were reported (Food and Drug Administration, 2006; Morbidity and Mortality Weekly Report, 2007). Of the 88 outbreak-associated *E. coli* O157:H7 cases in 2006, one outbreak associated with bagged fresh spinach accounted for 32, and two outbreaks associated with lettuce accounted for 14 cases (Food and Drug Administration, 2006). Some of the major outbreaks linked with *E. coli* O157:H7 are summarized in Table 2.1.

Table 2.1. Summary of major *E. coli* O157:H7 outbreaks occurred since 1982.

Period	Location	Source	Total No. of people became ill	Total No of people hospita-ized	Cases of HUS/HC	No of Death	Source
Aug-82	Oregon/ Michigan	Hamburger eaten at fast food chain	29				MMWR, November 1982
Jul-Aug, 90	North Dakota	Roast Beef	70	16	2		MMWR, April, 1991
Nov-92-Feb-93	Washington, Idaho, Nevada, California	Hamburger from fast food chain	>500	144	30	4	MMWR, April, 1993
Jan-93	Washington	Ground beef	203				MMWR, April, 1993

Table 2.1 Continued

Period	Location	Source	Total No. of people became ill	Total No of people hospita-ized	Cases of HUS/HC	No of Death	Reference
Nov-Dec, 94	Seattle King County, WA	Dry-cured Salami	20	3	1		MMWR, March, 1995
Jun-95	Georgia	Hamburger eaten at fast food chain	10	10			MMWR, March, 1996
Jul-95	Illinois	Swimming pool	12	12	3		MMWR, May, 1996
Oct-96	Northeast, United State	Odwalla brand Apple cider	45	28	12		MMWR, January, 1997

Table 2.1 Continued

Period	Location	Source	Total No. of people became ill	Total No of people hospita-ized	Cases of HUS/HC	No of Death	Reference
Jul-95	Illinois	Swimming pool	12	12	3		MMWR, May, 1996
Oct-96	Northeast, United State	Odwalla brand Apple cider	45	28	12		MMWR, January, 1997
Jul-97	Michigan and Virginia	Alfalfa sprouts	60	25	2		MMWR, August, 1997
Sep-99	Newyork	Waterborne	116	65	11	2	MMWR, Sept, 1999
Jun-98	Wisconsin	Cheese curd	55	25	55		MMWR, Octomber, 2000

Table 2.1 Continued

Period	Location	Source	Total No. of people became ill	Total No of people hospita-ized	Cases of HUS/HC	No of Death	Reference
Sep-06	Multistate	Spinach	183	95	29	1	MMWR, Sept, 2006

As *E. coli* O157:H7 outbreaks are often associated with ground beef, the USDA Food Safety and Inspection Service (FSIS) routinely analyses ground beef samples to determine the presence of *E. coli* O157:H7. A total of 26,521 raw ground beef samples were tested from FY2000 to FY2003, and 189 (0.71%) of the tested samples were positive for *E. coli* O157:H7 (Naugle *et al.*, 2005). In order to control bovine source-related *E. coli* O157:H7 infection, the FSIS issued notice to manufacturers of raw ground beef products in October 2002 that they must reassess their HACCP plans regarding this pathogen (MMWR, 2004). This effort resulted in a decline in cases of *E. coli* O157:H7 infection associated with ground beef. During FY2004, 7,294 ground beef samples were tested and 15 (0.2%) tested positive for *E. coli* O157:H7 (Naugle *et al.*, 2006). However, the frequency of isolation of *E. coli* O157:H7 in ground beef samples in 2005 and 2006

remained at the same level as of 2004 (Morbidity and Mortality Weekly Report, 2007). This data indicate that the prevalence of *E. coli* O157:H7 is at high rates in beef related products and better measures are still necessary to prevent its occurrence.

QUORUM SENSING AND AUTOINDUCER 2

Quorum sensing was first described in the regulation of bioluminescence in marine bacteria *Vibrio fischeri* and *Vibrio harveyi* (Bassler *et al.*, 1997). Both these organisms use autoinducer molecules for the production of bioluminescence. Enzymes required for the production of light are coded by the luciferase structural operon *luxCDABE* (Miller and Bassler, 2001)**.** In the *V. fischeri* quorum sensing system, LuxI produces autoinducer-1 (AI-1) molecules and binding of the AI-1 molecules to the receptor protein LuxR activates the transcription of *luxCDABE* operon genes (Miller and Bassler, 2001). The quorum sensing cascade in *V. harveyi* consists of a two-component circuit. In addition to AI-1 molecules, autoinducer-2 (AI-2) molecules are also involved in the regulation of the bioluminescence. A protein product of *luxS* is involved in the production of AI-2, whereas sensor proteins LuxP and LuxQ function together to detect AI-2 molecules (Bassler *et al.*, 1997).

Since the identification of QS in *V. fischeri* and *V harveyi*, a diverse group of bacterial species has been shown to possess different QS systems for the production and utilization of autoinducer molecules in population density-dependent controlled gene expression (Xavier and Bassler, 2003). Optimal levels of these cell signaling (autoinducer) molecules and the interaction of these molecules with regulatory proteins

are key steps involved in the coordination of gene expression. Different signaling molecules including AI-1, AI-2, AI-3, oligopeptides, indole, cyclic peptides, and the *Pseudomonas* Quinolone Signal have been previously described (Bansal *et al.*, 2007; Bassler *et al.*, 1997; McKnight *et al.*, 2000; Pillai and Jesudhasan, 2007). Among these signaling molecules, N-acyl homoserine lactones (AHLs), also called AI-1 molecules, are used by Gram-negative bacteria for intra-species communication, whereas furanosyl borate diester molecules, termed AI-2, are used for both inter and intra-species communication (Schauder *et al.*, 2001). AI-3 molecules of unknown structure have been shown to be used primarily in the bacterial-host communication, while autoinducer peptides (AIP) and short chain amino acids are known to be used by Gram-positive bacteria (MDowell *et al.*, 2001; Sperandio *et al.*, 2003). Among the different autoinducer molecules, AI-2 has been considered the universal signaling molecule, since the *luxS* gene, which is involved in the production of AI-2, is widely conserved among the different bacterial species including *V. harveyi, E. coli, and Salmonella* (Xavier and Bassler, 2003). The *luxS* gene is involved in the synthesis of AI-2 molecules by metabolically converting *S*-adenosine methionine (SAM) into *S*-ribosyl homocysteine (SRH). Fig. 2.1 shows the pathway for the production of AI-2 molecules in *E. coli* cells. In AI-2 biosynthesis, following the methyl transfer from *S*-adenosyl methionine to its various substrates, *S*-adenosylhomocysteine (SAH) is formed. The Pfs enzyme removes adenine from SAH to form *S*-ribosylhomocysteine (SRH) and LuxS acts on SRH to produce homocysteine and 4,5-dihydroxy-2-3 pentanedione (DPD). The reaction of

these unstable DPD molecules with water leads to cyclation and consequent formation of furanones known as AI-2 molecules (Schauder *et al.*, 2001).

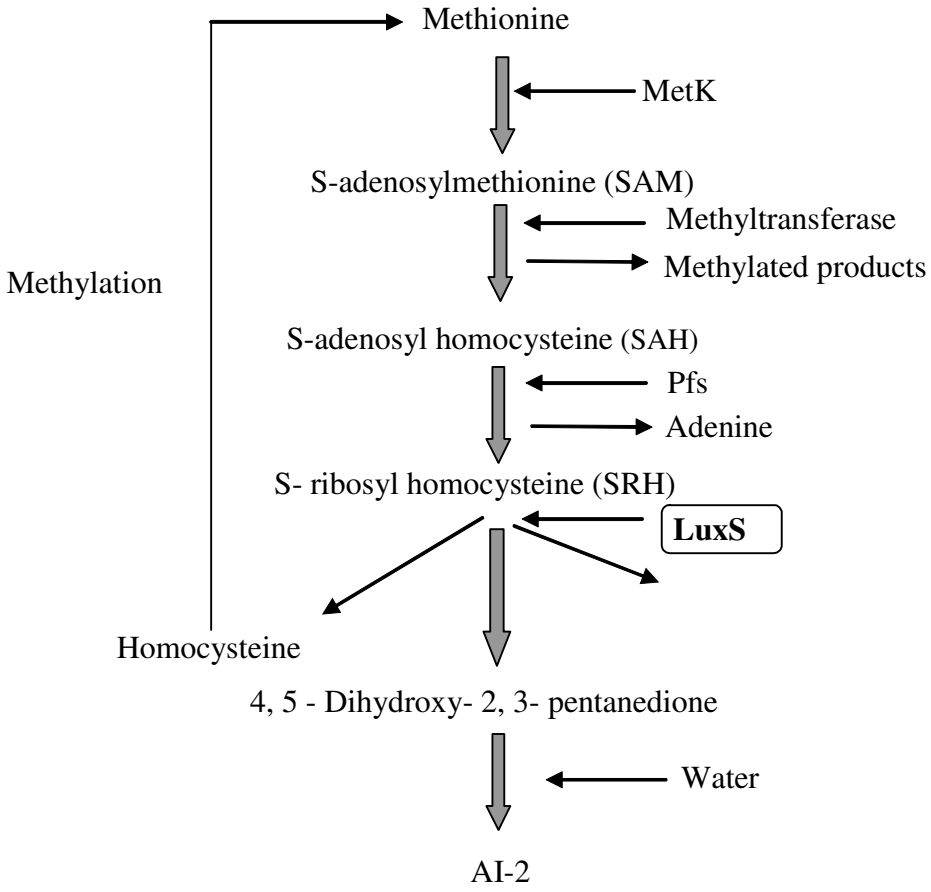

Fig. 2.1. Schematic representation of AI-2 production pathway in *E. coli* cells (Schauder *et al.*, 2001).

AI-2 molecules produced in the presence of LuxS synthetase then are utilized by bacterial cells as a signal transducer for the controlled gene and protein expression (Arevalo-Ferro *et al.*, 2003; Walters and Sperandio, 2006a). The channel proteins involved in AI-2 import inside the bacterial cells can vary depending on the type of bacterial species. The role of channel proteins involved in the uptake of AI-2 molecules in *V. harveyi* is well characterized (Bassler, 2002; Tu and Bassler, 2007). At low cell density the sensor protein autophosphorylates and transfers phosphates to LuxU, this in turn results in the phosphorylation of the response regulator, LuxO. Phosphorylation of LuxO then activates the expression of small regulatory RNA in conjugation with sigma 54 (Fig. 2.2). This small regulatory RNA along with chaperone Hfq binds to the activator region of protein LuxR and destabilizes it, which in turn puts the quorum sensing cycle in off condition. At high cell density AI-2 molecules bind to the sensor protein, LuxP, and this process switches kinase activity into phosphatase activity. The transfer of kinase to phosphatase activity results in the inactivation of LuxO and activation of LuxR for AI-2-controlled bioluminescence. In *E. coli*, *S. Typhimurium*, and other bacteria that possess the *lsr* operon, AI-2 molecules accumulated in the extracellular environment are internalized by the ABC transporter system encoded by *lsr* operon genes (Taga *et al.*, 2003) (Fig. 2.2). Four of the *lsr* operon genes namely *lsrA*, *lsrB*, *lsrC*, and *lsrD* are known to be involved in encoding the ABC transporter system (Li *et al.*, 2007; Taga *et al.*, 2003). The AI-2 uptake process in *E. coli* and *Salmonella* is controlled by kinase protein LsrK, which phosphorylates AI-2 molecules upon entry into the cell. It has been hypothesized that phosphorylated AI-2 molecules are responsible for

the inactivation of the *lsr* operon repressor protein LsrR and, thereby, activation of the *lsr* operon for AI-2 uptake.

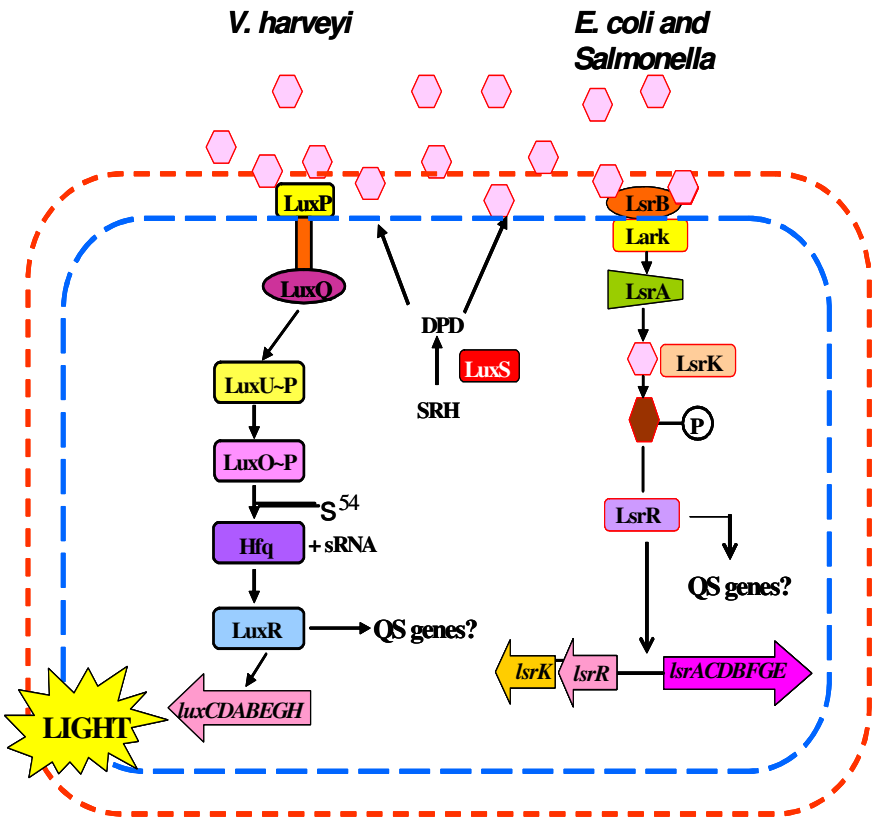

Fig. 2.2. Uptake and processing of AI-2 molecules in *V. harveyi*, *E. coli*, and *Salmonella* cells.

Miller et al. (2004) reported that AI-2 can exist in two different inter convertible forms based on the presence or absence of boron in the ring structure of AI-2 molecules (Miller *et al.*, 2004). In *V. harveyi*, LuxP protein contains a net positive charge that

favors the binding of conventional AI-2 molecules, which have a negative charge in the boron moiety, while in *S*. Typhimurium, LsrB protein contains a net negative charge and does not bind to boron moiety present in the conventional form of AI-2 molecules. Taga et al. (2001) and Xavier et al. (2005) have reported the uptake of AI-2 in spite of *lsrB* mutation and predicted the possibility of an alternative transport system in bacterial cells (Taga *et al.*, 2001; Xavier and Bassler, 2005). Recently, James et al. (2006) have suggested the role of *rbsB* as a transporter of AI-2 in *Actinobacillus actinomycetemocomitans*. Furthermore, studies performed in our laboratory suggest that *rbs* operon genes (*rbsB,* periplasmic binding protein; and *rbsK*, cytoplasmic kinase) were up-regulated in the presence of AI-2 activity (unpublished data). These results in combination suggest that *rbsB* (known to be the *luxP* homolog) may potentially be functioning for internalization of AI-2.

The production of AI-2 by LuxS has been thought to be a pivotal mechanism for bacterial environmental adaptation (McDougald *et al.*, 2003; Wen and Burne, 2004). However, recent studies suggest that LuxS acts as an important metabolic enzyme and AI-2 molecules synthesized by LuxS may be used as metabolites (Walters *et al.*, 2006; Winzer *et al.*, 2003). Microbial response in the presence of AI molecules can vary depending on the type of organisms. For example, the role of AI molecules in *E. coli* has been thought to up-regulate the expression of virulence factor-associated genes, whereas QS in *V. cholerae* down-regulates virulence genes to prevent continuing damage to host cells (Zhu *et al.*, 2002). In *Salmonella, hilA* is a central regulator of the genes involved in epithelial cell invasion. The presence of AI-2 molecules appears to

down-regulate expression of *hilA* and up-regulate expression of *hha,* which is a negative regulator of *hilA.* These results suggest that AI-2 may function as a negative regulator of virulence genes in *Salmonella.*

QUORUM SENSING IN *E. COLI*

Escherichia coli O157:H7 is an intestinal commensal organism of bovine animals and is responsible for a number of foodborne outbreaks (Rangel *et al.*, 2005)**.** In *E. coli* cells, LuxR receptor protein is responsible for binding with AI-1 molecules known to be involved in intra-species communication (Walters and Sperandio, 2006b). Although *E. coli* cells do not appear to have genetic components to synthesize AI-1 molecules, it contains the structural component SdiA, which is homologues to LuxR, a receptor for AI-1 (Ahmer, 2004; Michael *et al.*, 2001). Thus, *E. coli* cells have the ability to recognize AI-1 molecules. Like many other Gram-negative bacterial species, *E. coli* can produce AI-2-like molecules with maximum production at the late logarithmic growth phase (Surette and Bassler, 1998). Of the entire *E coli* genome, about 5-10% of the genes are controlled by AI-2-like molecules present in spent condition media (cell free supernatant) (DeLisa *et al.*, 2001; Sperandio *et al.*, 2001). AI-2 molecules are thought to play a critical role in biofilm formation of *E. coli* cells (Domka *et al.*, 2007; Gonzalez Barrios *et al.*, 2006; Moreira *et al.*, 2006).

Even though AI-2 molecules are known to regulate gene expression in *E. coli*, Sperandio et al (2003) demonstrated that, AI-3, rather than AI-2, is responsible for the control of genes involved in motility, flagella and typeIII secretion system in *E. coli*

O157:H7. In their study in vitro-synthesized AI-2 molecules failed to activate the expression of the LEE region and genes involved in motility and flagella movement, whereas the cell free supernatant (CFS) fraction (AI-3), which binds to the C-18 Sep Pack columns and extracted with methanol was responsible for the activation of genes involved in AE lesion formation, and flagella and motility (Fig. 2.3). QseA, a putative regulator of the LysR family, activates the transcription of a two-component system QseEF and *ler*, *and* thereby activating other genes of LEE regions involved in the formation of AE lesion formation (Fig. 2.3). Mutations in *qseA* or *ler* significantly reduce typeIII secretion activity in *E. coli* O157:H7 (Sperandio *et al.*, 2002). The two-component regulatory system called QseBC is known to control the genes for flagella action and motility (Sperandio *et al.*, 2002). In this regulatory system, *qseB* encodes for the response regulator, while *qseC* encodes for the sensor kinase.

In vitro analyses using epithelial cell lines have shown that the presence of cell signaling molecules leads to an increased level of attaching and effacing (AE) lesion formation in intestinal epithelial cells by activating the expression of genes present in the LEE pathogenicity island (Kim *et al.*, 2007; Sperandio *et al.*, 2001; Sperandio *et al.*, 2003). Several studies have shown that in the absence of *luxS* gene, which is necessary for AI production, bacterial cells show reduced adherence of bacterial cells to in vitro epithelial cell lines (Kim *et al.*, 2007; Sperandio *et al.*, 2003). The ability of AI-3 molecules to mimic the action of catecholamine such as epinephrine and nor-epinephrine further demonstrates that AI molecules may modulate bacterial response during interaction with host body (Bansal *et al.*, 2007; Sperandio *et al.*, 2003).

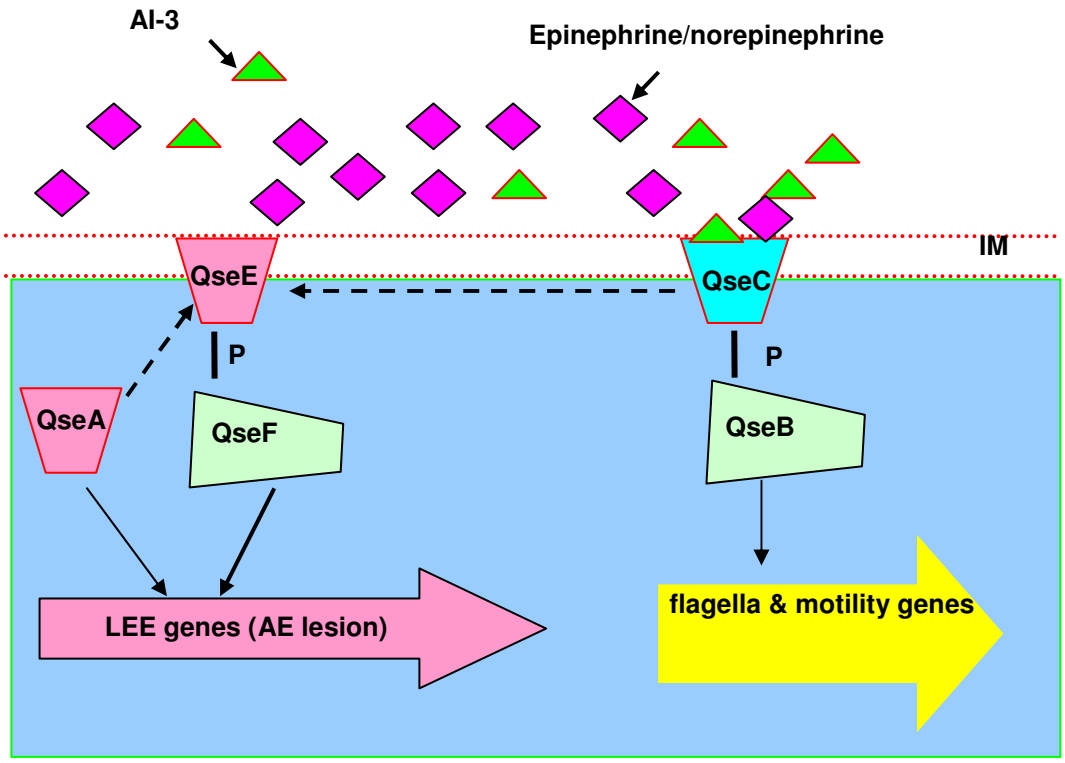

Fig. 2.3. Regulation of pathogenicity island LEE, and flagella and motility in *E. coli* O157:H7 are controlled by AI-3 and catecholamines such as epinephrine (epi) and nor-epinephrine (NE).

Recently some authors have attempted to understand the role of *luxS* and AI molecules using both in vitro and in vivo studies (Jeon and Itoh, 2007; Zhu *et al.*, 2007). Although both these studies showed the reduced adherence of *luxS* mutant strains to epithelial cells, no marked differences were observed between *luxS* mutant and parental wild type strains when in vivo studies were conducted using mice and rabbit models. These results suggest that the function of *luxS* gene was being restored during in vivo

analysis. The *luxS* gene involved in the production of AI molecules is present in the variety of Gram-positive and Gram-negative flora including those considered as commensal intestinal micro flora belonging to *Enterobacter* and *Enterococcus* genus (Xavier and Bassler, 2003). It is very likely that AI molecules produced by commensal microflora may be complementing the *luxS* mutant strain to up-regulate genes associated with virulence factors.

QUORUM SENSING IN RELATION TO FOODS

Cell-cell signaling occurs in a diverse group of microorganisms including those that are commonly associated with foods. Several authors have mentioned that the quorum sensing-mediated cell-cell signaling is likely to occur in both spoilage and pathogenic bacterial species when present in food matrices (Smith *et al.*, 2004). Since foods are complex ecosystems, the fate and behavior of microorganisms can be completely different in different food systems (Pillai and Jesudhasan, 2007). Therefore, when the role of autoinducer molecules is studied in context with food, the synergistic effect of the surrounding environment including the indigenous microflora present must be considered (Pillai and Jesudhasan, 2007; Smith *et al.*, 2004).

The dominant microflora of aerobically and vacuum packed meat products are *Pseudomonas* spp (~10^9 during spoilage) and *Enterobacteriaceae* (~10^6 to 10^7), respectively (Bruhn *et al.*, 2004; Liu *et al.*, 2006; Smith *et al.*, 2004). In *Enterobacteriaceae* family, both *Serratia proteamaculans* and *Hafina alvei* are dominant microflora in vacuum packed meat products. *S. proteamaculans* produces AI-1 (AHLs)

molecules and possesses the *lusI/luxR* homologues *sprI/spiI* (Christensen *et al.*, 2003). The function of *sprI/spiR* mediated cell signaling in *S. proteamaculans* is the controlled expression of *lipB* (Christensen *et al.*, 2003). The role of the LipB protein in the secretion of lipases and proteases is well established. Christensen et al. (2003) demonstrated the role of *Serratia proteamaculans* in milk curdling using a QS system (Christensen *et al.*, 2003). In their study a wild type strain of *S. proteamaculans* resulted in milk curdling after 18 h at room temperature; however no milk spoilage was noticed when the *sprI* mutant strain was inoculated in milk. The addition of AHL molecules in milk inoculated with a *sprI* mutant strain of *S. proteamaculans* also resulted in milk curdling suggesting that AHLs molecules may be involved in other food spoilage microorganism. Bruhn et al. (2004) demonstrated that *Serratia proteamaculans* can also utilize the AHLs (AI-1) molecules produced by *Hafina alvei*, which i the most commonly identified AHLs-producing bacterium in the food system (Bruhn *et al.*, 2004). The role of proteases produced by *P. fluorescens* has been implicated in food spoilage. Liu et al. (2006) showed that the AHL molecules produced by *P. fluorescens* are involved in the spoilage of milk and ground beef. In their study the addition of exogenous AHL molecules stimulated protease production in *P. fluorescens* and mutant cells lacking the ability to produce AHLs molecules were found to be defective in protease production. Rasch et al. (2005) demonstrated that the presence of AHL molecules is responsible for the spoilage of bean sprouts (Rasch *et al.*, 2005). They showed that spoilage of bean sprouts inoculated with the AHL-negative mutant was delayed compared to sprouts inoculated with the wild type *Enterobacteriaceae* strain

A2JM. *Yersinia enterocolitica* cells produced AHLs in fish and meat extracts, but failed to do so in Mexican salad, lettuce salad, cucumber, pork and beef extract (Medina-Martinez *et al.*, 2007). These results were consistent with the report of Lu et al. (2004) where fish extracts showed the presence of autoinducer molecules, whereas ground beef extracts did not.

Clock et al. (2002) studied the ability of *E. coli* O157:H7, *S*. Typhimurium, *Campylobacter jejuni*, and *Campylobacter coli* to produce AI-2 molecules in different food matrices such as milk, chicken broth, and apple juice. The production of AI-2 was evident in milk and chicken broth, but not in apple juice. The amount of AI-2 molecules produced was higher at 25°C and 30°C compared to 4°C. Maximal amount of AI-2 molecules were detected at the end of 24 h, whereas most of the AI-2 molecules disappeared at the end of 48 h. It is unclear why no detectable level of AI-2 production was observed in apple juice; however, Lu et al. (2004) suggested that certain food matrices possess compounds capable of inhibiting the expression of AI-2 activity. In *Erwinia carotovora,* the AI-1 molecules synthesized via the *carI-expI* genes are responsible for regulating the production of plant cell wall-degrading exoenzymes and, thereby, production of soft rot (Smadja *et al.*, 2004; Toth *et al.*, 2004). Toth et al. (2004) demonstrated that transgenic potato plants were more susceptible to *E. carotovora* infection in the presence of AI-1 molecules compared to its absence. AI-2-like activity present on the surfaces of tomatoes has been linked to the potential of enhancing the production of bacterial biofilms (Lu *et al.*, 2005). Lu et al. (2004) studied the effect of different food preservatives such as sodium acetate, benzoic acid, propionic acid, and

sodium nitrate on AI-2-like activity. The presence of these food preservative compounds inhibited the expression of AI-2-like activity about 75% to 99%, suggesting that these chemicals were interfering with cell-cell communications (Lu *et al.*, 2004).

From these available data it appears that quorum sensing may have a critical role in food spoilage and pathogenic microflora. Since quorum sensing is thought to control spoilage and virulent mechanisms in pathogenic bacteria, the use of anti-quorum sensing based pathogen-intervention strategies may have significant potential in near future.

INHIBITION OF QUORUM SENSING

The use of compounds or treatments that can eliminate or prevent the growth of pathogenic or unwanted organisms is a useful strategy in food preservation, preventing biofilm growth, reducing surface fouling and treating bacterial infection. However, when microorganisms encounter such treatments they undergo harsh selective pressure to develop resistance and as a result bacterial infections that were once treatable are now becoming untreatable. Furthermore, the use of antibiotic treatments targeted at eliminating harmful bacteria not only kills infectious organisms, but also eliminates the beneficial microflora. Due to these reasons it is imperative to use alternative treatment strategies that target pathogenic traits and the control the apparatus of virulence factors in microorganisms (Rasmussen and Givskov, 2006a). Interestingly several studies have shown that a variety of microbial processes including virulence are coordinately regulated at the gene expression level using QS molecules. This fact has led the interest

in finding compounds or mechanisms that can serve as potential inhibitors of quorum sensing controlled pathogenicity.

The main steps involved in the quorum sensing-regulated gene expression are the production of signaling molecules by signal generator proteins and recognition of signaling molecules by the receptor proteins. Obviously compounds with a property to prevent quorum sensing-controlled gene expression by interfering with signal generator, signaling molecules themselves, or receptor proteins should be considered as quorum sensing inhibitors. From available research it appears that the ideal quorum sensing inhibitors should be low molecular weight compounds, should not interfere with the growth of microorganisms and should not have toxic effects to the bacteria or eukaryotic host, should be stable in nature and show resistance to metabolic disposal by higher organisms (Rasmussen and Givskov, 2006a).

Screening and identification of quorum sensing inhibitory compounds have been of interest to many researchers and some authors have also attempted to elucidate the possible mechanism(s) by which quorum sensing inhibitors function. It is also imperative to conduct in-depth studies to determine the suitability of inhibitory compounds for therapeutic purposes. For example, previous studies have demonstrated that halogenated furanones produced by the *Delisea pulchra* are able to disrupt quorum sensing-regulated behaviors in many bacteria, however, it appears that the halogenated furanones may be too reactive and toxic for the treatment of bacterial infections in human (Hentzer and Givskov, 2003). Rasch et al. (2004) also suggested that even though furanones at 0.01 or 0.1 μM were able to show some level of reduced mortality in

rainbow trout caused by *Vibrio anguillarum*, a higher concentration of only 10 µM had a detrimental effect on rainbow trout. In the following subsections the previously identified quorum sensing inhibitors will be discussed based on their target specificity.

Inhibitors for the signal generator. Interfering with the ability of signal generator to produce autoinducer molecules can be a useful strategy to disrupt quorum sensing-regulated behaviors in bacteria. There are few examples of compounds that have shown the ability of inhibiting the production of AI-1 and AI-2 molecules by interfering with signal generator proteins. Substrate analogs such as butyryl- *S*-adenosyl methionine, 5'-methylthioadenosine, sinefungin, and D/L-S-adenosylhomocysteine have shown the ability to block the production of AI-1 molecules by the RhlI signal generator protein. AI-2 molecules are produced by the enzymatic activity of LuxS synthetase where S-ribosyl-L-homocysteine serves as a substrate. Alfaro et al. (2004) has synthesized substrate analogues, *S*-anhydroribibosyl-L-homocysteine and S-homoribosyl-L-cysteine. These compounds can bind to LuxS in a similar fashion to that of the substrate and, thereby, reduce or eliminate the production of AI-2 molecules. The ability of these compounds to inhibit AI-1 and AI-2 generator proteins is need to be tested using in vitro assays and in vivo analyses.

Modification or interference with signaling molecules. Interference with autoinducer molecules by chemical degradation or enzymatic destruction are other useful strategies for disrupting quorum sensing-regulated behaviors in bacteria. Yates et al. (2002) studied the effect of pH and temperature as a function of AI-1 activity. According to the results, it appears that an increase in pH the level above 7 causes the lactone ring to open

in the structure of AI-1 signal molecules. This process of lactone ring opening is termed "lactonolysis". The efficiency of the lactonolysis process also depends on the temperature and length of the acyl side chain in the structure of AI-1 molecules. Increasing temperature from 22 to 37 ºC accelerated the rate of lactonolysis. Increased in acyl side chain length decreases the rate of lactonolysis; however, it appears that for an efficient lactonolysis process the length of side chain in AI-1 structure should possess at least four carbons. Certain plants also possess specific mechanisms by which they increase the pH in response to the pathogenic plant organisms. For example, a recognizable response of plants to *Erwinia carotovora* infection is to increase the pH (~8.2 from original pH of ~ 6.2) of the apoplastic fluid around the site of infection (Byers *et al.*, 2002; Nachin and Barras, 2000). The alkaline condition created by increased pH can prevent the expression of quorum sensing controlled genes. The use of strategies targeted at accelerating or facilitating ring opening could be of tremendous commercial value. However, the major drawback in controlling the lactonolysis process is that irrespective of how ring opening occurred (alkaline pH or enzymatic degradation), this process is reversible at acidic pH (Yates *et al.*, 2002).

Production of the AHL lactonase enzyme, AiiA, by the genus of *Bacillus* family has been shown to lower the level of active AI-1 signals by catalyzing ring opening (Dong *et al.*, 2000; Wang *et al.*, 2004). *Bacillus* itself does not produce AHL molecules and uses small peptides as a cell signaling molecule for controlled gene expression. Therefore, it is possible that the activation of *aiia* gene by *Bacillus* may provide a competitive edge over other Gram-negative microflora that utilizes AHLs molecules for

controlled gene expression. In *Erwinia carotovora,* the AI-1 molecules synthesized via the *carI-expI* genes are responsible for regulating the production of plant cell wall-degrading exoenzymes and, thereby, production of soft rots (Byers *et al.*, 2002). When a plasmid carrying the *aiiA* gene was introduced into *Erwinia carotovora,* it resulted in reduced virulence against potato, eggplants, and transgenic tobacco plants (Dong *et al.*, 2000; Dong *et al.*, 2004). The *aiiA* gene-carrying plant bacterium *Pseudomonas fluorescens* was also able to prevent soft rot production induced in potatoes by *Erwinia carotovora* and crown gall disease in tomatoes caused by *Agrobacterium tumefaciens* (Rasmussen and Givskov, 2006a). Several bacterial species such as *Pseudomonas, Arthrobacter, Agrobacterium, and Klebsellia* are known to possess homologous of AiiA (Park *et al.*, 2003; Uroz *et al.*, 2003). It appears that many bacteria can counteract AI-1-induced changes by possessing enzymes that can degrade the activity of AI-1 molecules. In addition to enzyme AiiA, the acyl chain-opening enzyme AiiD has been also found to modulate AHL structure by cleaving acyl side chains (Lin *et al.*, 2003). When *aiid* was expressed in *P. aeruginosa* it showed reduced accumulation of AHL molecules and, thereby, decreased expression of virulence factors and attenuated virulence against *Caenorhabditis elegans*.

Interference with the function of receptor proteins. The use of natural or synthetic biomimic of autoinducer molecules, which can interact with the receptor proteins is another approach to design novel drug formulation targeted at disrupting the quorum sensing processes in bacteria. Modified derivatives of AI-1 molecules such as incorporation of cyclo alcohol or cyclo ketone in the carbon side chain have been shown

to reduce the expression of LasR-controlled *lasI-gfp* fusion in *P. aeruginosa* (Smith *et al.*, 2003). Different derivatives of AI-1 molecules created by substitution of a sulphur moiety in the acyl chain of AI-1 molecules were also successful in blocking the expression of AI-1 receptor proteins LuxR and LasR (Persson *et al.*, 2005). Rasmussen et al. (2005b) also identified secondary metabolites, patulin, produced by *Penicillium coprobium* as an inhibitor of AI-1-based cell signaling in *P. aeruginosa.*. Western blot analysis performed with antibodies against the receptor protein LuxR showed that presence of patulin decreases the amount of LuxR protein. Compounds such as 4-nitro-pyridine-N-oxide, indole, *p*-benzoquinone, and 3-nitrobenzene sulphone amide are also able to serve as quorum sensing inhibitors even though they are not structurally related to autoinducer molecules (Rasmussen *et al.*, 2005a). Furthermore, using the *E. coli*-established LuxR quorum sensing system it was shown that 4-nitro-pyridine-N-oxide may possess inhibitory activity by interfering with AI-1 receptor proteins. When GeneChip-based transcriptome analysis was performed, 4-nitro-pyridine-N-oxide was able to lower the expression by 37% quorum sensing-regulated genes in *P. aeruginosa.* Production of halogenated furanones by the algae *Delisa pulchara* and the use of synthetic furanones have shown the ability to interfere with both AI-1-and AI-2-mediated QS systems (McDougald *et al.*, 2007; Ren *et al.*, 2004b). Manefield et al. (2002) hypothesized that inhibitory activity produced by furanones is due to the modulation of autoinducer receptor protein. Using western blot analysis they showed that the half-life of the LuxR protein was reduced up to 100-fold in the presence of furanones. Furthermore, using DNA microarray analysis Hentzer et al. (2003) showed

that furonones are able to repress virulence factor produced by *P. aeruginosa* and also increases the bacterial biofilm susceptibility to tobramycin and sodium dodecyl sulphate. Using mice model, Wu et al. (2004) demonstrated that synthetic furanones inhibit bacterial quorum sensing and enhance clearance of *Pseudomonas aeruginosa* in mice lung. Microarray analysis has also revealed that the pattern of up or down regulation for approximately 79% of the quorum sensing-regulated genes in *E. coli* can be reversed in the presence of synthetic furanones (Ren *et al.*, 2004b).

Quorum sensing inhibitors present in food. Cell-cell signaling occurs in a diverse group of microorganisms including those that are commonly associated with foods. Several authors have mentioned that the quorum sensing mediated cell-cell signaling is likely to occur in both spoilage and pathogenic bacterial species when present in food matrices (Pillai and Jesudhasan, 2007; Smith *et al.*, 2004). Since foods contain a multitude of components, it is also of growing interest to determine if food possesses compounds capable of interfering with quorum sensing activity.

In this context many meat products including ground beef and poultry meat are capable of inhibiting AI-2-like activity when assayed using a *V. harveyi* reporter strain (Lu *et al.*, 2004). Using hexane extraction and gas chromatography, AI-2 inhibitors present in both ground meat and poultry meat were also identified and characterized as medium and long chain fatty acids (Widmer *et al.*, 2007). When different fatty acids such as palmitic acid (C 16:0), stearic acid (C 18:0), oleic acid (C 18:1 ω9), and linoleic acid (C 18:2 ω6) were tested for AI-2 inhibition at varying concentrations (1mM, 5mM, and 10mM) that do not impede the growth of reporter strain, AI-2 inhibition ranging

from 25% to 90% was observed. Lu et al. (2004) also studied the effect of different food preservatives such as sodium acetate, benzoic acid, propionic acid, and sodium nitrate on AI-2-like activity. The presence of these food preservative compounds inhibited the expression of AI-2-like activity about 75% to 99%, suggesting that these chemicals were interfering with cell-cell communications.

Choo et al (2006) using reporter strain *Chromobacterium violaceum* showed that vanilla beans also possess compounds that can inhibit AI-1-induced violacein and these inhibitory factors of AI-1 can be extracted using non-polar solvent chloroform (Choo *et al.*, 2006). Bosgelmez-Tinaz (2007) also extracted inhibitory factors for AI-1 from the plant S*corzonera sandrasica* using chloroform (Bosgelmez-Tinaz *et al.*, 2007). These inhibitory factors were able to inhibit AI-1-induced violacein in *Chromobacterium violaceum* and quorum sensing-regulated carbapenem antibiotic production in *E. carotovora*. Interestingly inhibitory factors from both vanilla beans and S*corzonera sandrasica* were extracted using chloroform as a solvent suggesting that inhibitory factors may have some similarity in nature. However, none of these studies identified the chemical structure or nature of these compounds. Secondary plant metabolites such as phytochemicals are well known for their health benefits. Vattem et al, (2007) tested effect of bioactive dietary phytochemicals derived from different herbs, fruits and spices on cell signaling. Interestingly these phytochemicals extracts were able to show inhibition of AI-1 induced violacein in the *C. violaceum* reporter strain and swarming motility in *P. aeruginosa.*

CHAPTER III

IDENTIFICATION OF GROUND BEEF-DERIVED FATTY ACID INHIBITORS OF AUTOINDUCER-2 (AI-2)-BASED CELL SIGNALING*

OVERVIEW

Autoinducer-2 (AI-2) molecules are used by several microorganisms to modulate various bacterial processes including bioluminescence, biofilm formation, and virulence expression. Previous research has shown that certain food matrices including that of ground beef extracts possess compounds capable of inhibiting AI-2 activity. In the present study we have identified and characterized these AI-2 inhibitors from ground beef extract using hexane solvent extraction and gas chromatography (GC) analysis. Gas chromatographic analysis revealed the presence of several fatty acids such as palmitic acid (C 16:0), stearic acid (C 18:0), oleic acid (C 18:1 ω9), and linoleic acid (C 18:2 ω6) that were capable of inhibiting AI-2 activity. These fatty acids were tested (using the *Vibrio harveyi* BB170 and MM32 reporter strains) at different concentrations (1mM, 5mM, and 10mM) to identify differences in the level of AI-2 activity inhibition. AI-2 inhibition ranging from 25% to 90% was observed. A mixture of these fatty acids

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*Reprinted with permission from "Identification of Ground Beef-Derived Fatty Acid Inhibitors of Autoinducer-2 (AI-2)-Based Cell Signaling" by Kamlesh Soni, Palmy Jesudhasan, Martha Cepeda, G. K. Jayaprakasha, B. Patil, Michael Hume and Suresh D. Pillai, 2008, *Journal of Food Protection* (in press).

(prepared at equivalent concentrations to those present in the ground beef extract) showed between 52 and 65 % inhibition of AI-2 activity. The fatty acid mixture was also found to negatively influence *E. coli* K12 biofilm formation. These results demonstrates that both medium and long chain fatty acids in ground beef have the ability to interfere with AI-2 based cell signaling.

INTRODUCTION

Studies have shown that a variety of microbial processes such as growth, sporulation, toxin production, virulence, antibiotic synthesis, and motility in bacterial cells are coordinately regulated at the gene expression level by a variety of intra-and inter-cellular *autoinducer* molecules in a process termed quorum sensing (QS) (Sperandio *et al.*, 2003; Surette and Bassler, 1998; Taga and Bassler, 2003). Different autoinducer (AI) molecules including AI-1, AI-2, AI-3, oligopeptides, indole, cyclic dipetides, and the *Pseudomonas* Quinolone Signal have been previously described (Bansal *et al.*, 2007; McKnight *et al.*, 2000; Pillai and Jesudhasan, 2007, 2007; Sperandio *et al.*, 2003; Taga and Bassler, 2003). Among these autoinducer molecules, autoinducer 1 (AI-1) is thought to be highly species-specific (Cao *et al.*, 2001), while autoinducer 2 (AI-2) is thought to serve as a 'universal' bacterial signal for inter-species communication (Bassler, 1999; Schauder *et al.*, 2001). Autoinducer 3 (AI-3) is thought to be involved in communication between the bacterial cells and the host's signaling system (Sperandio *et al.*, 2003). Among the different AI molecules, AI-2 has been considered the universal signaling molecule since the *luxS* gene, which is involved in the

production of AI-2, is widely conserved among the different bacterial species (Xavier and Bassler, 2003).

New information about the bacterial species that possess QS systems, the levels of auto-inducer molecules within the host bacterial cells, the genes that are controlled by QS, and the identity of the signaling molecules continues to be obtained (Ahmer *et al.*, 1998; Bruhn *et al.*, 2004; Miller *et al.*, 2004; Sperandio *et al.*, 2003). However, very little is known about how cell signaling influences spoilage and pathogenic bacteria in foods (Pillai and Jesudhasan, 2007; Smith *et al.*, 2004). We have previously shown that certain foods such as fresh ground beef and fresh poultry meat contain compounds capable of inhibiting AI-2 activity (Lu *et al.*, 2004). The focus of this study was to identify and characterize the compounds present in ground beef extracts that interfere with AI-2 based cell signaling.

MATERIALS AND METHODS

Preparation of aqueous ground beef extracts. Autoinducer bioassay (AB) medium was prepared as described earlier (Lu *et al.*, 2004). Commercially purchased ground beef (~15% fat content) was homogenized with an equal volume (1w:1v) of autoinducer bioassay (AB) medium in a stomacher (Seward Stomacher® 400 LAB System, Norfolk, UK) for 2 min. The homogenate was centrifuged (10,000 X *g*; 10 min), and the supernatant was collected and filter-sterilized using a 0.2-µm filter.

In vitro-synthesis of AI-2. The AI-2 molecule was synthesized in our laboratory based on a previously published protocol (Schauder *et al.*, 2001; Sperandio *et al.*, 2003).

Briefly, His-tagged LuxS and Pfs (proteins required for the formation of AI-2) were isolated and purified using a nickel resin column (Qiagen Inc., Valencia, CA). The purified enzymes were incubated with 1 mM S-adenosyl homocysteine (Sigma-Aldrich, St. Louis, MO) for 1 h at 37° C, and AI-2 was further separated from the enzymes using a centrifuge column (Biomax-5, Millipore, Billerica, MA). AI-2 activity was confirmed using *Vibrio harveyi* BB170 reporter strain (Lu *et al.*, 2004).

AI-2 activity inhibition assays. *V. harveyi* reporter strains BB170 (*lux*N::Tn5) (kindly provided by Dr. Bonnie Bassler, Princeton University), and MM32 (*luxN*::cm *luxS*::Tn*5*) (ATCC # BAA-1121), which exhibits bioluminescence in the presence of AI-2 molecules, were used to screen for AI-2 activity. The experimental method to determine inhibition of AI-2 activity was based on our earlier report (Lu *et al.*, 2004). Briefly, 90 µl of the freshly diluted (1:5000) culture of the reporter strain in autoinducer bioassay (AB) medium were mixed with 5 µl of in vitro synthesized AI-2 and 5 µl of the test samples in a 96-well plate. Negative control (10 µl of AB media) and positive controls (5 µl of in vitro AI-2 + 5 µl of AB media) were included in each experiment. Six replicates were used for each measurement. The microtiter plates were incubated at 30°C with moderate shaking (100 RPM) and the luminescence response of the reporter strains was monitored using a Wallac 1420 plate reader (PerkinElmer, Shelton, CT). Inhibition of AI-2 activity was expressed as a percentage relative to the corresponding positive control and was calculated as: 100 – [(light unit measurement of sample/light unit measurement of positive control) x 100] (Lu *et al.*, 2004).

Identification and characterization of inhibitory compounds. Identification and characterization of inhibitory compounds present in the beef extracts were based on a solvent extraction procedure. Equal volumes (1 liter) of ground beef extracts (prepared as described earlier) were mixed with varying polarity solvents such as hexane (minimal polarity), ethyl acetate (medium polarity), and methyl ether ketone (highest polarity), followed by rigorous mixing (200 rpm) for 1 h and a solvent phase separation for 30 minutes. The solvents were evaporated off in a chemical hood. The extracts were dissolved (1 mg/ml) in sterile water (80ºC) and were analyzed for AI-2 inhibition. When assayed, the hexane extracted material showed the highest level of AI-2 inhibition, suggesting that the inhibitory compounds may be hydrophobic in nature. The remained aqueous content of hexane-ground beef extract was also checked for AI-2 inhibitions. The GC analysis of the hexane extracts were performed as described earlier (Hossen, 2005). The GC system consisted of a Varian Model 3400 equipped with a split injector, a flame ionization detector, and a fused silica capillary column Supelco SP 2560 (100 m, 0.25 mm I.D., 0.20 ìm film, Bellefonte, PA). Briefly, methyl esters of the fatty acids were generated using hydrogen as a carrier gas and the extracted fatty acid esters were injected into a GC system with a flame ionization detector to pass through a fused silica capillary column. Fatty acid concentrations of the hexane solvent extracted materials were determined using standard AOCS official method (Ce 1h-05) under conditions described by Hossen and Hernandez (Hossen, 2005). Reference fatty acid methyl esters were used in identifying and quantifying the fatty acids present in the sample.

Inhibition of AI-2 activity by selected fatty acids. The fatty acids (palmitic (C 16:0), stearic (C 18:0), oleic (C 18:1 ω9), and linoleic (C 18:2 ω6) that accounted for major proportion in GC analysis were commercially purchased (Sigma, St. Louis, MO) and tested at different concentrations (1mM, 5 mM, and 10 mM) using *V. harveyi* (BB170 and MM32) reporter strains to screen for inhibition of AI-2 activity. These fatty acids (C 16:0, C 18:0, C 18:1 ω9, and C 18:2 ω6) were also mixed together in water in the following proportion (7.16, 3. 71, 9.97, 0.58 µg /ml or 0.027, 0.013, 0.035, 0.002 mM, respectively) to obtain a 1X concentration. The 1X concentration was based on the stoichoimetric relationships of these fatty acids obtained during the GC analysis. Additionally, 10X and 100X mixtures were also prepared and their inhibitory effect on AI-2 activity was also measured. To check if the observed inhibition of AI-2 is due to acid functionality of selected fatty acids, portion of the 100X fatty acids mixture (pH 5.9) was neutriled using 0.1N NaOH solution and tested for AI-2 inhibition. To rule out the possibility that observed inhibition of AI-2 activity was due to a bactericidal effect on the reporter strains, portions of the bioassays were plated on Luria-Marine (LM) media when an inhibition of AI-2 activity inhibition was observed. The plates were incubated at 30° C, and *V. harveyi* colonies were enumerated after 24 h of incubation.

Effect of fatty acids on E. coli K-12 biofilm formation. The biofilm formation assay was based on a previous report (Ren *et al.*, 2005). Briefly, *E. coli* K12 cells were grown overnight in Luria- Bertani (LB) medium and 95 µl of the freshly diluted (1:100) cultures were mixed with 5 µl of test samples in a sterile round-bottom 96-well polystyrene plate (Nunclon^{TM} surface, Denmark). A positive control (95 µl culture + 5 µl

of DI water) was included for each experiment. The plates were incubated at 37°C for 48 h without shaking. At the end of the incubation period, the unattached cells were removed and the plates were washed with distilled water. The remaining biofilm was stained for 15 min using 100 µl of a 1% (w/v) crystal violet solution. Crystal violet-stained cells were solubilized with 200 µl of an ethanol: acetone (80:20) solution for quantifying the formed biofilms. The samples from each well (125 µl) were pipetted to a new flat-bottom 96-well plate (Corning Inc. Corning, NY), and OD_{570} nm readings were recorded. To rule out the possibility of bactericidal effect of the fatty acids on the *E. coli* K-12 cells, *E. coli* cells were grown for 24 h in the presence of the ground beef extract as well as the mixture (100X) of fatty acids. After incubation, the cultures were serially diluted and plated for enumeration.

Statistical analysis. Six replicates were included for the AI-2 inhibition and biofilm assays. The experiments were repeated at least twice. Statistical analyses (paired t-test) were performed using SPSS version 12.0 (Chicago, Ill).

RESULTS

Solvent extraction of the ground beef extract. The inhibitory compounds present in the beef extract were extracted using a solvent extraction procedure and by employing varying polarity solvents such as hexane, ethyl acetate, and methyl ether ketone. When the different ground beef-solvent extracts were tested for inhibition of AI-2 activity, the hexane extracts showed the highest (95%) level of AI-2 inhibition (Fig. 3.1). There was no difference in the plate counts of the *V. harveyi* BB170 reporter strain, confirming that

the observed inhibition of AI-2 activity was not an experimental artifact due to reduced viability of the reporter strain (Table 3.1). When tested, the aqueous content of hexane-ground beef extract did not result in appreciable amount of AI-2 inhibition (18%).

Identification of fatty acids by GC analysis. GC analysis of the hexane extracts showed that C 18:1 ω9 (41.6%), C 16:0 (29.9%), and C 18:0 (15.5%) acids were the primary fatty acids and their concentrations were 9.97, 7.16, and 3.71 μg/g; respectively (Table 3.2). Additionally, linoleic, myristic, palmitoleic, linolenic, and pentadecanoic acids were also found to be present at varying concentrations in the hexane extracts from the ground beef sample (Table 3.2).

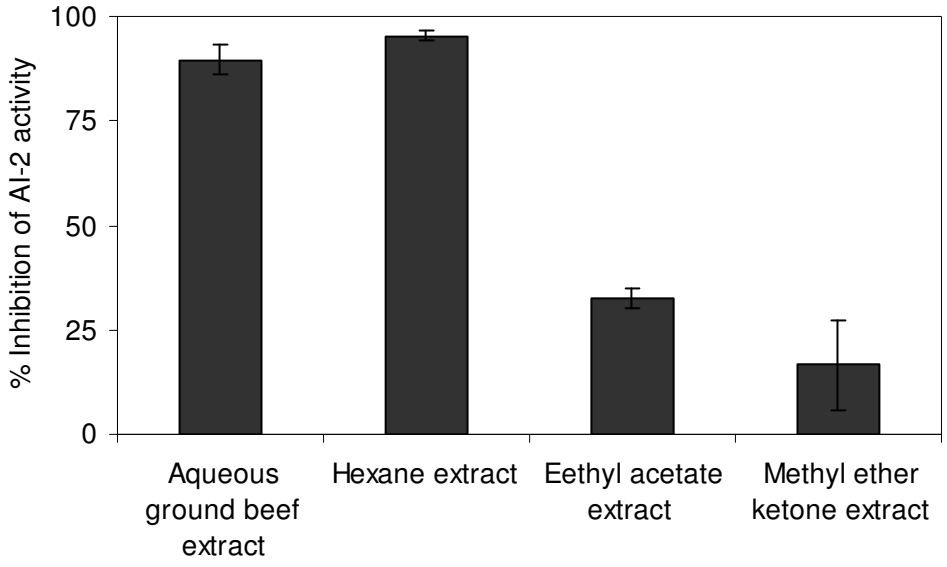

Fig. 3.1. Inhibition of AI-2 activity (based on *Vibrio harveyi* BB170 reporter strain) by aqueous ground beef extracts solvent extracts. Y-axis represents % inhibition of AI-2 activity (± standard error, n = 6).

Table 3.1. Log CFU/ml of *V. harveyi* and *E. coli* cells with different treatments.

Bacterial Strain	Treatment	Log CFU/ml
V. harveyi *BB170*	Positive control	7.1 ± 0.1
	Negative control	7.1 ± 0.2
	Ground beef-hexane extract	7.2 ± 0.2
	10mM Linoleic acid (C 18:2 ω6)	7.1 ± 0.2
	10mM Palmitic acid (C 16:0)	7.4 ± 0.3
	10mM Oleic acid (C 18:1 ω9)	7.4 ± 0.2
	10mM Stearic acid (C 18:0)	7.4 ± 0.2
	Mixture of fatty acids (100X)	7.2 ± 0.2
V. harveyi MM32	Positive control	7.3 ± 0.1
	Negative control	7.3 ± 0.2
	10mM Linoleic acid (C 18:2 ω6)	7.7 ± 0.2
	10mM Palmitic acid (C 16:0)	7.6 ± 0.3
	10mM Oleic acid (C 18:1 ω9)	7.4 ± 0.2
	10mM Stearic acid (C 18:0)	7.2 ± 0.2
	Mixture of fatty acids (100X)	7.4 ± 0.2
E. coli K12	Control	8.5 ± 0.3
	Ground beef extracts	8.7 ± 0.1
	Mixture of fatty acids (100X)	8.7 ± 0.1

Log CFU/ml (± standard error, n = 3) of the *V. harveyi* (BB170 and MM32) reporter strain at the end of the AI-2 inhibition bioassay. Log CFU/ml (n = 3) of *E. coli* cells in LB plates at the end of 24 hrs.

Table 3.2. Composition of fatty acids based on GC analysis of the hexane extract.

Fatty Acid	Percentage (%)	Concentration (μg /g)
Linoleic (C 18:2 ω6)	2.46	0.58
Myristic (C 14:0)	4.68	1.12
Oleic (C 18:1 ω9)	41.59	9.97
Palmitic (C 16:0)	29.88	7.16
Palmitoleic (C 16:0)	3.93	0.94
Stearic (C 18:0)	15.50	3.71
Linolenic (C 18:3)	0.63	0.15
Pentadecanoic (C 15:0)	1.33	0.31

Inhibition of AI-2 activity by fatty acids. Linoleic, oleic, palmitic, and stearic acids were tested for their ability to inhibit AI-2 activity using the *V. harveyi* (BB170 and MM32) reporter strain. The fatty acids even at varying concentrations (1mM, 5 mM, and 10 mM) exhibited some level of inhibition and the level of AI-2 inhibition increased as the concentration of fatty acids increased (Fig. 3.2, Fig. 3.3). Oleic acid (C 18:1 ω9) in general showed higher AI-2 inhibition compared to linoleic (C 18:2 ω6), palmitic (C 16:0), and stearic acids (C 18:0). Rather than testing these fatty acids singly, mixtures of these fatty acids were prepared (1X, 10X, and 100X) and were tested for their ability to inhibit AI-2 activity. Using *V. harveyi* BB170 as a reporter strain, inhibition of AI-2 activity averaged 64.5 (± 5.2) %, 81.5 (± 4.7) %, and 86.6 (± 4.9) %, respectively, for the

1X, 10X and 100X solutions (Fig. 3.4). Furthermore, when *V. harveyi* MM32 was used as a reporter strain, AI-2 inhibition averaged 52.3 (± 3.9) %, 74.4 (± 4.1) %, and 91.3 (± 8.2) % respectively for the 1X, 10X, and 100X solutions (Fig. 3.4). Statistical analysis (*paired t-test*) showed that the reporter strain had no significant effect on the level of AI-2 inhibition at a particular fatty acid mixture concentration. Inhibition of AI-2 activity was higher at 10X concentration compared to 1X, whereas no differences were observed between 10X and 100X concentrations. Furthermore, level of AI-2 inhibition did not differ significantly between 100X fatty acid solutions (% AI-2 inhibition 86.6 ± 4.9) and neutralized 100X fatty acid solution (% AI-2 inhibition 98.6 ± 8.7) suggesting that observed AI-2 inhibition is not due to acid functionality of fatty acids. Bacterial counts of the BB170 and MM 32 reporter strains (in the positive, negative and fatty acids treatments) averaged between 7.1 to 7.6 log CFU/ml (Table 3.1), suggesting that the growth of the reporter strains was not influenced by the fatty acids concentrations used in this study.

Influence of fatty acids on E. coli K-12 biofilm formation. Fig. 3.5 shows the influence of fatty acids on biofilm formation by *E. coli* K12 cells. The addition of inhibitory compounds from the aqueous extracts or fatty acids mixture resulted in 2 to 4-fold reduction in biofilm formation as compared to the positive control. There was no difference in the *E. coli* K-12 plate counts in the presence of ground beef extracts or fatty acid compared to the positive control implying that cell growth was not affected by the experimental treatments (Table 3.1).

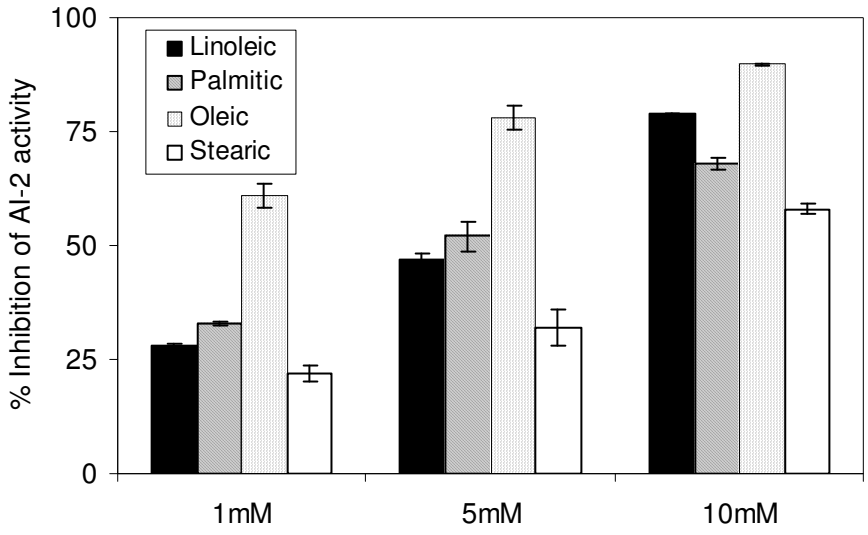

Fig. 3.2. Inhibition of AI-2 activity by linoleic, palmitic, oleic, and stearic acids based on *Vibrio harveyi* BB170 reporter strain. Y-axis represents % inhibition of AI-2 activity (± standard error, n = 6).

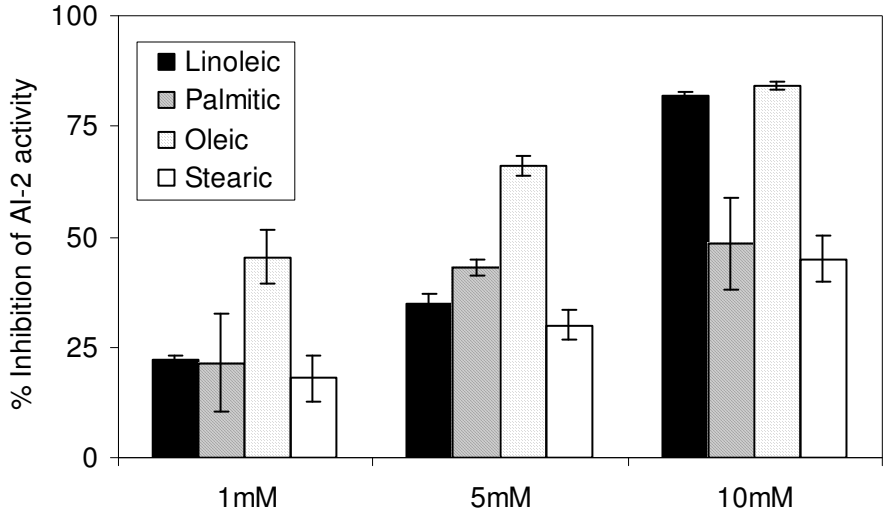

Fig. 3.3. Inhibition of AI-2 activity by linoleic, palmitic, oleic, and stearic acids based on *Vibrio harveyi* MM32 reporter strain. Y-axis represents % inhibition of AI-2 activity (± standard error, n = 6).

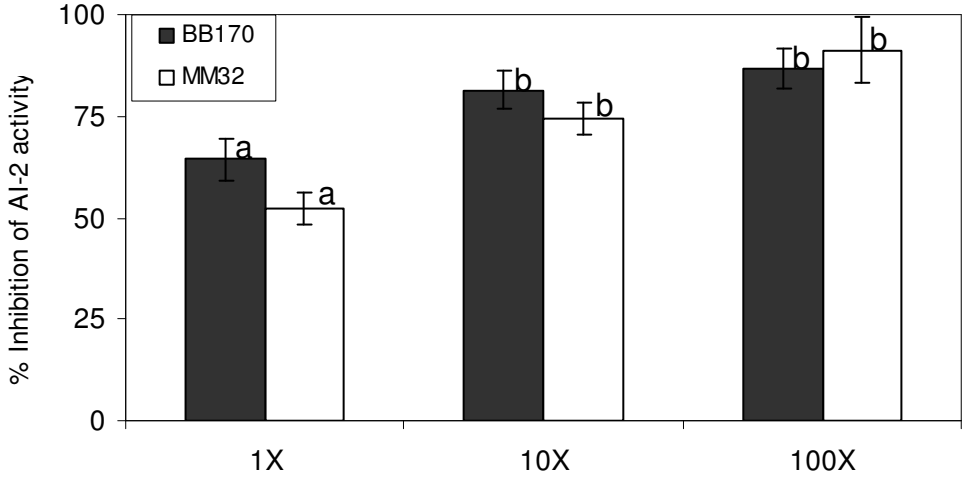

Fig. 3.4. Mean levels of inhibition of AI-2 activity by fatty acid mixture using *Vibrio harveyi* BB170 and MM32 as reporter strains. Here, Y-axis represents % inhibition of AI-2 activity (± standard error, n = 6), and 1X mixture of fatty acids= 0.58, 9.97, 7.16, and 3. 71 µg/ml of C 18:2 ω6, C 18:1 ω9, C 16:0, and C 18:0 acids; respectively. Bars with a similar small cap letter indicate no significant differences based on paired t- test ($p \geq 0.05$).

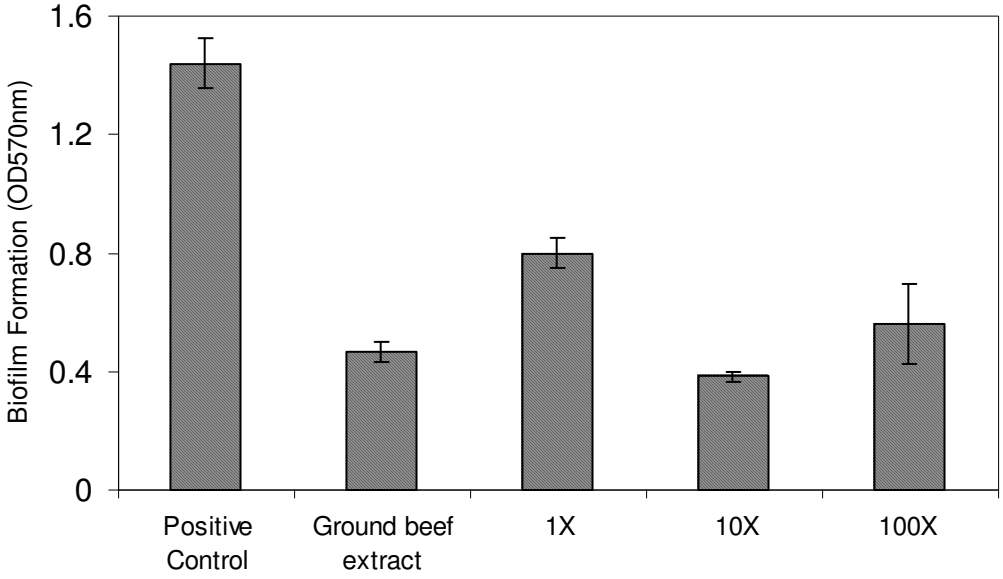

Fig. 3.5. Reduced biofilm formation of *E. coli* K12 cells in the presence of AI-2 inhibitory compounds. Y- axis represents mean biofilm formation (± standard error, n = 6) measured at OD570nm.

DISCUSSION

Screening and identification of quorum sensing inhibitory compounds from natural and man-made source is of continuing interest (Lu *et al.*, 2004; Rasmussen *et al.*, 2000; Rasmussen and Givskov, 2006a; Ren *et al.*, 2004a). Lu et al (2004) have reported that some food matrices, including ground beef, are capable of inhibiting AI-2-like activity (Lu *et al.*, 2004). Halogenated furanones produced by the alga, *Delisea pulchara,* and synthetic furanones have exhibited the ability to interfere with both AI-1- and AI-2- mediated QS systems (Manefield *et al.*, 2002; Ren *et al.*, 2004b). Microarray

analysis has revealed that approximately 79% of the QS regulated genes in *E. coli* were repressed by the presence of synthetic furanones (Ren *et al.*, 2004b). Lu et al. (2004) studied the effect of different food preservatives such as sodium acetate, benzoic acid, propionic acid, and sodium nitrate on AI-2-like activity (Lu *et al.*, 2004). These preservatives inhibited AI-2 activity between 75% to 99%.

In this study, we have identified and characterized some of the specific fatty acids in ground beef that are responsible for the inhibition of AI-2 activity. These fatty acids inhibited AI-2 activity when tested either individually (1mM, 5mM and 10mM) or in a mixture at varying strengths (1X, 10X, 100X) (Fig. 3.1, 3.2 and Fig. 3.3). Furthermore, ten fold increases (1X to 10X, and 10X to 100X) in fatty acid concentrations did not result in a corresponding ten-fold decrease in AI-2 activity suggesting that there might be point of "saturation" in the inhibitory activity. The GC analysis of the hexane extracts yielded eight different fatty acids. The inhibition observed by the aqueous extract was 88% compared to the hexane extract (95%) and the 1X mixture (65%) of the four different fatty acids (Fig. 3.1, Fig. 3.4). This variation in the inhibition levels is not surprising because the hexane extract contains a high concentration of inhibitors. We do acknowledge that the hexane extract may contain inhibitors in addition to what was identified by the GC analysis. It must be pointed the fatty acids used in this study were among the predominant fatty acids that were extracted. Saturated and unsaturated fatty acids such as caprylic and linoleic acid have been reported in the literature to possess antimicrobial activity (Pierre and Ryser, 2006). However, the concentrations that have been used in the previous studies were much

higher than what was tested in this study (Marounek *et al.*, 2003; Pierre and Ryser, 2006). The inhibitory effect on AI-2 activity of the fatty acids observed in the present study is significant because our results indicate that these fatty acids are able to modulate AI-2 based cell signaling without affecting bacterial cell viability.

Microbial biofilm formation has been linked to AI-2 based cell signaling and has been implicated in pathogenicity (Domka *et al.*, 2006; Herzberg *et al.*, 2006; Labbate *et al.*, 2004; Rasmussen and Givskov, 2006b; Ren *et al.*, 2005). The *E. coli* K12 strain used in this study has been known to produce AI-2 molecules, and has been used by other investigators to study QS processes (Domka *et al.*, 2006; Ren *et al.*, 2004b; Wang *et al.*, 2005b). Importantly, biofilm formation in *E. coli* K12 cells has been reported to be under the direct control of AI-2 based cells signaling (Domka *et al.*, 2006; Herzberg *et al.*, 2006). Our results suggest that a mixture of C 16:0, C 18:0, C 18:1 ω9, and C 18:2 ω6 which was capable of interfering with biofilm formation has value in controlling biofilm formation. Higher concentrations of the inhibitory fatty acids above those concentrations found in ground beef did not result in greater inhibition biofilm formation suggesting that there might be saturation point for the amount of inhibitory activity. Like fatty acids, furanones which have been shown to inhibit biofilm formation are now considered to be of clinical significance. Synthetic furanones are now undergoing extensive clinical trials as a therapeutic agent against cystic fibrosis associated biofilms (Wu *et al.*, 2004b). We are currently attempting to delineate the specific mechanism(s) by which the fatty acids inhibit AI-2 activity and inhibit biofilm formation.

In conclusion, these results demonstrate that both medium chain and long chain fatty acids isolated from ground beef have the ability to inhibit AI-2 based cell signaling and biofilm formation. Further research, however, will be needed to fully understand the mode of action and potential suite of applications for these fatty acids as AI-2 inhibitors in maintaining food quality and food safety. Such studies can lead to the development of high value-added products from ground beef which can be utilized to enhance the safety and quality of foods.

CHAPTER IV

PROTEOMIC ANALYSIS TO IDENTIFY THE ROLE OF LUXS/AI-2 MEDIATED PROTEIN EXPRESSION IN *ESCHERICHIA COLI* O157:H7*

OVERVIEW

Microorganisms employ autoinducer molecules to modulate various bacterial processes including virulence expression, biofilm development, and bioluminescence. The universal autoinducer molecule AI-2 is hypothesized to mediate cell signaling in *E. coli* O157:H7. We investigated the role of AI-2 on the *E. coli* O157:H7 cellular proteins using a 2D gel electrophoresis-based proteomic approach. The protein expression patterns between two experimental comparisons were studied namely, a) a wild type *E. coli* O157:H7 and its isogenic *luxS* mutant, and b) the *luxS* mutant and the *luxS* mutant supplemented with AI-2 molecules. Eleven proteins were differentially expressed between the wild type and the *luxS* mutant strain, whereas 18 proteins were differentially expressed in the *luxS* mutant strain when supplemented with AI-2. The tryptophan repressor binding protein (WrbA), phosphoglycerate mutase (GpmA), and a putative protein YbbN were found to be differentially expressed under both experimental comparisons. The FliC protein which is involved in flagellar synthesis and motility was

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up-regulated in the wild type strain but was not influenced by the addition of synthetic AI-2 molecules to the *luxS* mutant suggesting the involvement of signaling molecules other than AI-2 on flagellar synthesis and motility.

INTRODUCTION

Bacterial cells utilize autoinducer molecules for cell-cell communication in a process termed quorum sensing (Camilli and Bassler, 2006; Lu *et al.*, 2004; Pillai and Jesudhasan, 2007). Autoinducer molecules play a key role in bacterial metabolism, virulence, and sporulation (Hardie *et al.*, 2003; Sperandio *et al.*, 2003; Zhao *et al.*, 2006). Autoinducer AI-2 has been considered the universal signaling molecule since it is recognized by several bacterial species (Camilli and Bassler, 2006). The *luxS* gene, which is involved in the production of AI-2, is widely conserved among different bacterial species including *Escherichia coli* (Xavier and Bassler, 2003). The LuxS synthase is involved in the production of AI-2 molecules as part of the S- adenosyl methionine (SAM) degradation pathway.

E. coli O157:H7 infection is a major cause of food-borne illness around the world (Moxley, 2004). Studies are focused on delineating the role of autoinducer molecules such as AI-2 molecules in *E. coli* O157:H7 regulation (DeLisa *et al.*, 2001; Hardie *et al.*, 2003; Ren *et al.*, 2004b; Sperandio *et al.*, 2001; Wang *et al.*, 2005a). There is also a growing interest in understanding how autoinducer molecules, pathogens and foods interacts (Cloak *et al.*, 2002; Lu *et al.*, 2004; Medina-Martinez *et al.*, 2007). Sperandio and coworkers suggest that *E. coli* O157:H7 senses two classes of signals to

activate its virulence and motility genes, namely AI-3, and a host-derived catecholamine class of hormones (Sperandio *et al.*, 2003). DNA microarray analysis using cell-free supernatants has been used to identify the role of autoinducer molecules in various cellular processes of *E. coli* (DeLisa *et al.*, 2001; Ren *et al.*, 2004b; Sperandio *et al.*, 2001). Recently, Kim et al. (2007) also used cell-free supernatants and a proteomic approach to understand the role of AI-2-like activity in *E. coli* virulence regulation (Kim *et al.*, 2007). The objective of this study was to use in vitro synthesized AI-2 molecules and proteomic analysis to identify the specific role(s) of LuxS/AI-2 mediated signaling in *E. coli* O157:H7.

MATERIALS AND METHODS

Preparation and quantification of AI-2 molecules. In vitro synthesized AI-2 was prepared and quantified as described previously (Sperandio *et al.*, 2003). Briefly, His-tagged LuxS and Pfs (proteins required for the formation of AI-2) were isolated and purified using a nickel resin column (Qiagen Inc., Valencia, CA). The purified enzymes were incubated with 1 mM S-adenosyl homocysteine (Sigma-Aldrich, St. Louis, MO) for one hour at 37° C, and AI-2 was further separated from the enzymes using a centrifuge column (Biomax-5, Millipore, Billerica, MA) and its concentration determined.

Bacterial strain and growth conditions. *E. coli* O157:H7 wild type strain (86-24) and its isogenic *luxS* mutant (VS-94) strains (provided by V. Sperandio, Southwestern Medical Center, Dallas, TX) were used in this study. The *luxS* mutant strain lacks the

capacity to synthesize LuxS protein and thereby lacks the ability to synthesize AI-2 molecules. The strains were grown at 37°C at 100 rpm in LB medium supplemented with 0.5% glucose until an OD_{600} of 1.2 was attained (which occurred within 3 h). In vitro synthesized AI-2 (25 µM/ml) was added to LB broth from the beginning of growth period to expose the *luxS* mutant *E. coli* O157:H7 cells to an exogenous source of AI-2.

AI-2 activity measurement. AI-2 activity was measured as Relative Light Units (RLU) using the *V. harveyi* BB 170-based AI-2 activity bioassay (Lu *et al.*, 2004). The RLU was calculated based on the ratio of the bioluminescence of the test sample to the negative control. The wild type cells produced a significant amount (139 ± 3.7 RLU) of AI-2 activity at the late-log phase (OD_{600} ~1.2) compared to the *luxS* mutant cells which failed to produce the luminescence in the reporter strain. The LB medium supplemented with 25 µM/ml of AI-2 produced an activity of 120 ± 7.5 RLU.

Soluble protein extraction. Bacterial strains were grown OD_{600} (~1.2) in triplicate for each experimental treatment (the wild type, the *luxS* mutant, and the *luxS* mutant grown with 25 µM/ml AI-2), and the soluble protein fractions were extracted from each sample independently using B-Per® bacterial protein extraction reagent (Pierce, Rockford, IL). The Ready Prep™ 2-D cleanup-kit (Bio-Rad, Hercules, CA) was used to reduce the ionic contaminants in the protein preparation. The purified proteins were dissolved in 100 µl of rehydration buffer [9.5 M urea, 2% w/v CHAPS, 18 mM DTT, 0.5% amphyolytes and one tablet of protease inhibitor (Roche Diagnostic, Mannheim, Germany)], and insoluble proteins and cell debris were removed by centrifugation.

Two-dimensional gel electrophoresis. Protein concentrations were measured using the Bradford protein assay kit (Pierce, Rockford, IL). Two hundred micro liters (µl) of Bradford reagents were mixed with 1.7 µl of the protein samples and absorbance was measured at 580 nm. Preliminary studies using immobilized pH gradient (IPG) strips in the range of pH 3-10 indicated that the majority of the soluble proteins were detectable in the pH 4-7 range. Hence for subsequent analysis, IPG strips in the pH 4-7 range were employed. Protein loads of 35 µg in 125 µl of rehydration buffer and 800 µg in 250 µl of rehydration buffer were used for 7 cm and 13 cm IPG strips (pH 4-7) respectively. The IPG strips were re-hydrated overnight in a rehydration tray (Bio-Rad, Hercules, CA).

For the first dimensional electrophoresis, the isoelectric focusing of 7 cm IPG strips were conducted at a linear voltage gradient with ~15000 final V-hrs (250 V in 15 min, 4000 V in 2 hrs, 10000 V in 2.5 hrs, and holding at constant 500 V/hr) using Protean IEF cell (Bio-Rad, Hercules, CA). For 13 cm IPG strips, the isoelectric focusing was conducted in linear mode to achieve ~65000 final V-hrs (Amershan Bioscience, Piscataway, NJ). After the required V-hr was applied, the IPG strips were incubated for 15 min in equilibration buffer I [6M urea, 30% glycerol, 2% SDS (w/v), 50 mM Tris-HCl (pH 8.8), 1% dithiotheritol, and approximately 5mg of bromophenol blue] followed by 15 min in equilibration buffer II [6M urea, 30% glycerol, 2% SDS (w/v), 50 mM Tris- HCl (pH 8.8), 2.5 % iodoacetamide, and bromophenol blue as color indicator]. Second dimension electrophoresis was performed at 150 constant volts using 10% SDS-PAGE gel. Protein spots were visualized using Sypro Ruby fluorescence stain

(Molecular Probe, Eugene, OR) for the 7 cm IPG strip gel. Gelcode® blue stain reagent (Pierce, Rockford, IL) was used to stain the 13 cm IPG strip gel.

Data analysis. The extracted and purified proteins from three experimental replicates of each treatment (wild type, *luxS* mutant, *luxS* mutant grown with 25 µM/ml AI-2) were run in duplicate using 7 cm IPG strips, resulting in six independent gels for each treatment. Additional 2D gels were run from the 13 cm IPG strips to assist in spot excision. The gels were scanned using Gel Doc (Bio-Rad, Hercules, CA) and the raw images were analyzed using advanced PDQuest 2-D gel analysis software version-8 (Bio-Rad, Hercules, CA). The protein expression patterns of; (i) the wild type *E. coli* O157:H7 against the *luxS* mutant (an AI-2 deficient strain), and (ii) the *luxS* mutant against the *luxS* mutant grown with AI-2 molecules; were compared. Only those spots with spot intensities exhibiting ±1.5-fold change difference were short-listed for identification (Arevalo-Ferro et al., 2005).

In-gel proteolytic digestion and MALDI-TOF. The protein spots of interest were manually excised (approximately 1 mm in size) and placed in a 96-well microtiter plate for in-gel digestion. The collected gel plugs were followed by their in-gel digestion as described elsewhere (Wilm et al., 1996) with little modifications. Briefly, washing and dehydration of gel plugs were conducted using solution-A [25 mM of ammonium bicarbonate (ABC)] and solution-B [2:1 acetonitrile (ACN):50 mM ABC]. Fifty microliter of Solution A and B were exposed alternatively 3 times to gel plugs for 2 and 5 minutes respectively in rotary shaker (100 rpm) with subsequent removal of complete supernatant after each exposure. Finally the spots were dried for 1 hr at room

temperature before subjecting to proteolytic digestion. Proteolytic digestion was performed using trypsin (Promega, WI, USA) prepared at 20 μg mL^{-1} in 25 mM ABC and incubating at 37˚C overnight.

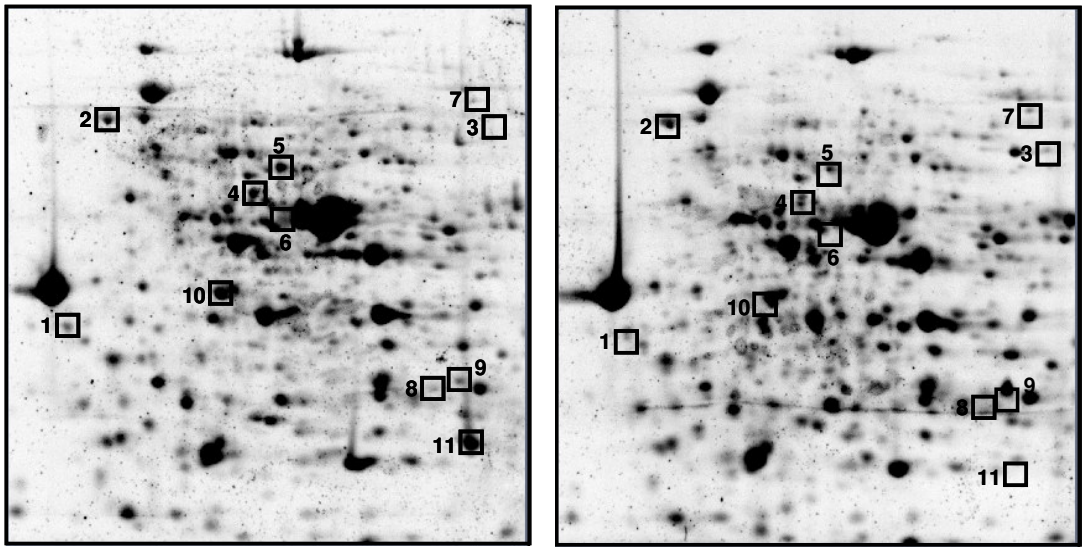
The digested samples were spotted onto MALDI targets using a ProMS™ (Genomic Solutions, Ann Arbor, MI) robot capable of sample clean-up prior to MS analysis. All MALDI-MS experiments were performed using a model 4700 Proteomics Analyzer MALDI-TOF/TOF (Applied Biosystems, Foster City, CA) instrument. The MS data for each gel spot was acquired using the reflectron detector and twenty tandem MS spectra per spot were acquired. All MS and MS/MS data were queried against the Swiss-Prot protein sequence database using the GPS Explorer (Applied Biosystems) software. The parameters for database searching were as follows: taxonomy, *Escherichia coli*; database, Swiss Prot; enzyme, trypsin; maximum missed cleavages, 1; variable modifications, oxidation (Met); peptide tolerance, 85 ppm; and MS/MS fragment tolerance, 0.3 Da. To verify the reproducibility of MALDI-MS data, ten spots were reanalyzed.

Motility assay. Motility assays were performed using motility agar media (Acumedia, Baltimore, ML) to validate the expression of specific proteins observed in the proteomic data. In vitro synthesized AI-2 molecules (25 μM/ml) were added to motility agar at around 37°C prior to pouring the plates. Late-log phase culture (~10^9 cfu/ml) of wild type and *luxS* mutant *E. coli* O157:H7 cells were inoculated in the center of motility agar plates using sterilized toothpicks. The plates were incubated for 16 h at 37°C, and ability

of the cells to swim in semisolid motility agar medium was recorded by measuring the diameter of the formed halos.

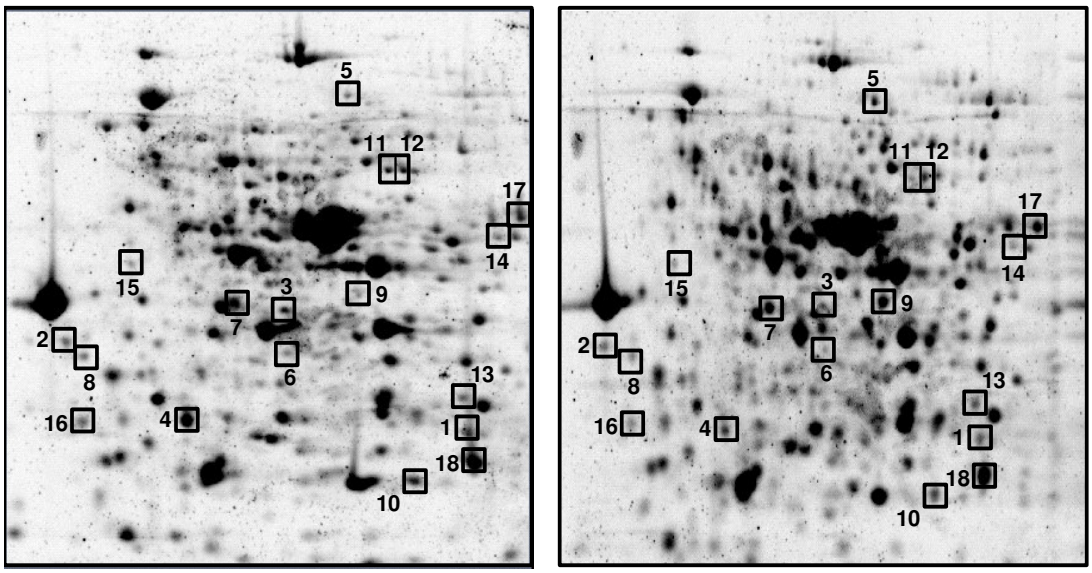
RESULTS

A total of 312 protein spots were detectable across the different experimental treatment groups (Fig. 4.1 and Fig. 4.2). The reproducibility of the individual 2-D gels was evaluated by correlation coefficient analysis. Average correlation coefficient among individual gel comparisons of different treatment groups were > 0.7, suggesting high similarity in spotting pattern (Bland *et al.*, 2006). Since each treatment had 6 gels, protein spots appearing in at least 4 out of a 6 gels were short-listed for further analysis.



AI-2 deficient *luxS* mutant strain Wild type strain

Fig. 4.1. Differentially expressed protein due to the presence or absence of *luxS* gene.



AI-2 deficient *luxS* mutant strain

luxS mutant strain amended with AI-2

Fig. 4.2. Differentially expressed protein spots in *luxS* mutant in the presence of AI-2.

When spots exhibiting significant changes on intensity (± 1.5-fold) were identified using MALDI-MS, they resulted in protein score confidence interval between 95% and 100% (Choe *et al.*, 2005).

Influence of *luxS* mutation on protein expression in *Escherichia coli* O157:H7 strain. The differentially expressed proteins between the wild type and *luxS* mutant strain are shown in Fig. 4.1 and Table 4.1. Eleven proteins were identified as differentially expressed. Among these, 5 proteins were up-regulated and 6 proteins were down-regulated. Proteins associated with carbohydrate metabolism (GpmA, TalB, FumB, and Mak) were affected by the *luxS* mutation. The *gpmA* and *talB* gene products

which catalyze reactions in the glycolysis pathway were up-regulated and down-regulated respectively in the wild type as compared to the *luxS* mutant strain. The *fumB* gene product (malate dehydratase) involved in the interconversion of malate to fumarate during the TCA cycle was up-regulated in the wild type strain. The results also indicate that proteins related to amino acid metabolism (PepD), cellular processes and signaling (YbbN, FliC, and YbhF), protein biosynthesis (TufA), nucleotide transport and processing (DeoA), and yet to be fully characterized protein (WrbA) were also affected by the *luxS* mutation.

Influence of AI-2 on protein expression in *Escherichia coli* O157:H7 strain. The differentially expressed proteins between the *luxS* mutant and *luxS* mutant supplemented with AI-2 are shown in Fig. 4.2 and Table 4.2. Eighteen proteins (4 up-regulated and 14 down-regulated) were differentially expressed. The proteins related to carbohydrate metabolism (PfkA, Eda, Eno, FruB, and GpmA), and amino acid metabolism (SpeE, NikA, and IscC), were affected by presence the of AI-2 molecules. Proteins related to cellular processes and signaling (YbbN, UspE, Tsx, and GlmS), and some yet-to-be fully characterized proteins (SspB, YnjE, and WrbA) were also influenced by the presence of AI-2 molecules.

Motility assays. The wild type cells produced larger halos (25.1 ± 1.9 mm) in the motility agar compared to that of *luxS* mutant (19.8 ± 0.8 mm) or when the *luxS* mutant was supplemented with AI-2 (18.7 ± 0.9 mm) (Fig. 4.3).

Table 4.1. Differentially expressed proteins in the wild type strain compared to *luxS* mutant strain of *E. coli* O157:H7.

ID[a]	PI[b]	Mr[b]	Protein Identification	Gene	Fold difference*	Function
			Cell signaling and processes			
1	4.5	28	Protein ybbN	*ybbN*	-2.8	Posttranslational modification, protein turnover, chaperones
2	4.7	62	Flagellin	*fliC*	1.5	Cell motility
3	6.3	75	Hypothetical ABC transporter ATP-binding protein	*ybhF*	3.7	Defense mechanisms
			Metabolism			
4	5.2	45	Thymidine phosphorylase	*deoA*	-4	Nucleotide transport and metabolism
5	5.3	55	Aminoacyl-histidine dipeptidase	*pepD*	-2.1	Amino acid transport and metabolism

Table 4.1 Continued

ID[a]	PI[b]	Mr[b]	Protein Identification	Gene	Fold difference*	Function
6	5.2	28	Transadolase B	*talB*	-1.8	Carbohydrate transport and metabolism
7	6.4	80	Fumerate hydratase class I, anaerobic	*fumB*	2.7	Energy production and conversion
8	6.1	19	Probable manno kinase	*Mak*	1.8	Metabolism
9	6.2	20	2,3 biphosphoglycerate dependant phosphoglycerate mutase	*gpmA*	2.3	Carbohydrate transport and metabolism
			Cellular Processing			
10	5.4	45	Elongation factor (EF-Tu)	*tufA*	-2.3	Protein biosynthesis

Table 4.1 Continued

ID[a]	PI[b]	Mr[b]	Protein Identification	Gene	Fold difference*	function
				Others		
11	6.2	18	Trp repressor binding protein	*wrbA*	-11	Repressor binding protein

a. ID refers to spot shown in Fig. 4.1,

b. Isoelctric point (*pI*) and molecular weight (*MW*) obtained in the experiment (practical).

* Fold difference in the protein expression of wild type strain compared to *luxS* mutant strain

Table 4.2. Differential protein expression in *luxS* mutant strain by AI-2 molecules.

ID[a]	PI[b]	Mr[b]	Protein Identification	Gene	Fold difference*	Function
			Cell signaling and processes			
1	4.5	28	Protein ybbN	*ybbN*	-2.8	Posttranslational modification, protein turnover, chaperones
2	4.7	62	Flagellin	*fliC*	1.5	Cell motility
3	6.3	75	Hypothetical ABC transporter ATP-binding protein	*ybhF*	3.7	Defense mechanisms
			Metabolism			
4	5.2	45	Thymidine phosphorylase	*deoA*	-4	Nucleotide transport and metabolism
5	5.3	55	Aminoacyl-histidine dipeptidase	*pepD*	-2.1	Amino acid transport and metabolism

Table 4.2 Continued

ID[a]	PI[b]	Mr[b]	Protein Identification	Gene	Fold difference*	Function
6	5.3	27	Sperimidine synthetase	*speE*	-2.7	Amino acid transport and metabolism
7	5.2	28	Gluthathione synthetase	*gshB*	-2.3	Coenzyme transport and metabolism
8	4.6	26	3-mercaptopyruvate sulphurtransferase	*sseA*	-2.2	Inorganic ion transport and metabolism
9	5.7	35	6-phosphofruktokinase isozyme I	*pfkA*	7.3	Carbohydrate transport and metabolism
10	6	18	4-hydroxy-2-oxoglutarate adolase	*eda*	-2.2	Carbohydrate transport and metabolism

Table 4.2 Continued

ID[a]	PI[b]	Mr[b]	Protein Identification	Gene	Fold difference*	Function
11	5.8	60	Nickel binding periplasmic protein binding precursor	*nikA*	-2.4	Amino acid transport and metabolism
12	5.8	58	Enolase	*Eno*	2.1	Carbohydrate transport and metabolism
13	6.2	20	2,3 biphosphoglycerate dependent phosphoglycerate mutase	*gpmA*	1.8	Carbohydrate transport and metabolism
14	6.2	45	Cystein desulfurase	*iscC*	-3	Amino acid transport and metabolism
15	4.8	39	Fructose specific IIA/FPr component (EIIA-Fru)	*fruB*	-2.6	Carbohydrate transport and metabolism

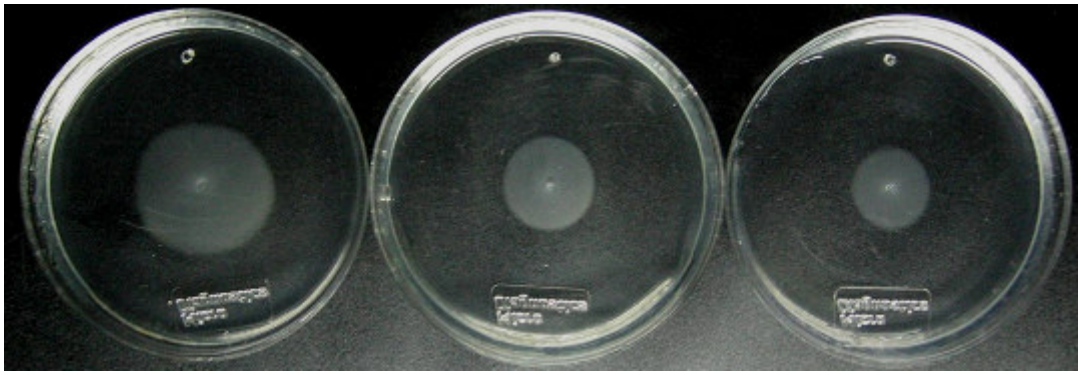
Table 4.2 Continued

ID[a]	PI[b]	Mr[b]	Protein Identification	Gene	Fold difference*	Function
				Others		
16	4.6	18	Stringent starvation protein	*sspB*	-3.2	Stress response
17	6.3	46	Putative thiosulphate sulfurtransferase ynjE precursor	*ynjE*	-2.6	Putative protein
18	6.2	20	Trp repressor binding protein	*wrbA*	-1.8	Repressor binding protein

a. ID refers to spot shown in Fig. 4.2.

b. Isoelctric point (*pI*) and molecular weight (*MW*) obtained in the experiment (practical).

* Fold difference in the protein expression of *luxS* mutant supplemented with AI-2 compared to the *luxS* mutant strain



Wild Type *luxS* mutant *luxS* mutant + AI-2

Fig. 4. 3. Motility halos produced by wild type and *luxS* mutant strain (in the presence or absence of AI-2 molecules).

DISCUSSION

The PepD (aminoacyl-histidine dipeptidase) protein is involved in the synthesis of amino acids such as alanine, aspartate, arginine, proline, and histidine synthesis. Brombacher et al. (2003) reported that repression of *pepD* gene is necessary for better growth of biofilm (Brombacher *et al.*, 2003). Gonzalez et al. (2006) has reported that biofilm formation is linked to quorum sensing (Gonzalez Barrios *et al.*, 2006). In this study, we observed a 2-fold down regulation of PepD in the wild type (as compared to the *luxS* mutant). The down regulation of *pepD* gene was observed in microarray analysis (Sperandio *et al.*, 2001). The results reported in this study in combination with these previous studies confirm the linkage between quorum sensing and biofilm formation via the involvment of the PepD protein. We observed the flavoprotein WrbA being down-regulated 11-fold in the wild type compared to the *luxS* mutant (Table 4.1).

This means that the WrbA protein is over-expressed in the *luxS* mutant. The tryptophan repressor TrpR is involved in regulating tryptophan biosynthesis whereas WrbA acts as a tryptophan repressor binding protein (Gorman and Shapiro, 2005). Though the exact biological role of WrbA has not yet been clearly identified, it has been reported that binding of WrbA with TrpR improves the stability between the TrpR and operator region of the Trp operon thereby repressing biosynthesis of tryptophan (Han and Lee, 2006). This suggests that in the *luxS* mutant the tryptophan biosynthesis is repressed due to the over-expression of WrbA. This repression could explain the altered metabolic pathways in *luxS* mutants that have been reported by previous investigators (Walters and Sperandio, 2006b; Winzer *et al.*, 2002).

We noticed that polyamine biosynthesis protein (SpeE) was repressed (-2.2 fold) in the presence of AI-2 (Table 4.2). Krin et al. (2006) also noticed the repression of *speE* gene by AI-2 molecules in *Photorhabdus luminescens* which also harbors the LuxS/AI-2 system(Krin *et al.*, 2006). We observed repression of universal stress protein UspE (-2.2 fold), and stringent starvation protein SspB (-3.2 fold) in the presence of AI-2 molecules (Table 4.2). Kim et al. (2007) noticed down-regulation (-3.0 fold) of another universal stress protein UspG in *E. coli* O157:H7 cells in the presence of cell-free supernatant containing AI-2 like molecules (Kim *et al.*, 2007). Microarray analysis has shown the repression of universal stress proteins in *Porphyromonsa gingivalis* by cell signaling molecules (Yuan *et al.*, 2005). These results imply that if AI-2 is present, the bacterial cell does not perceive the presence of stress factor(s) and consequently the stress response proteins are down-regulated. It is only in the absence of AI-2 either due to low

cell numbers or when nutrients are depleted (when AI-2 is rapidly metabolized) that the stress response is activated.

The two component system, QseBC, is known to regulate genes for flagella and motility using quorum sensing molecules. Protein FliC is involved in flagellar synthesis and is regulated by a QseBC system in EHEC (Sperandio *et al.*, 2003). The FliC protein was up-regulated in the wild type (Table 4.1) and addition of AI-2 molecules to the *luxS* mutant did not show change in FliC expression (Table 4.2). The motility assay results were in agreement with the protein expression data in that the addition of AI-2 molecules in motility agar medium did not influence the size of motility halo formed by *luxS* mutant strain. Sperandio et al. (2003) claim that flagellar and motility genes are up-regulated not by AI-2, but rather another autoinducer molecule, AI-3. The 3 proteins (WrbA, YbbN, GpmA) were present across the 2 experimental comparisons (LuxS mutation and presence of extraneous AI-2) (Table 4.1 and 4.2) indicating that AI-2 molecules produced by *luxS* play a specific role in the expression of these proteins. The *gpmA* gene product belongs to phosphoglycero mutase family and is involved in carbohydrate transport and metabolism (Turlin *et al.*, 2006). We observed 2.3-fold increase in GpmA protein expression in the wild type compared to the *luxS* mutant (Table 4.1). Furthermore, a 1.8-fold increase in GpmA levels was observed when the *luxS* mutant was supplemented with synthetic AI-2 (Table 4.2). Delisa et al. (2001) reported the higher expression (3.2-fold) *gpmB* which codes for a protein belonging to the phosphoglycero mutase family when *luxS* mutant strain was supplemented with CFS containing AI-2 like molecules. Compared to the 11-fold down-regulation of WrbA

protein in the wild type (Table 4.1), we observed only 1.8-fold down regulation of *luxS* mutant when supplemented with AI-2 molecules (Table 4.2). This difference in expression levels of WrbA among these two experimental comparisons highlights two possibilities namely, 1) WrbA expression is more actively controlled by *luxS* gene itself than AI-2 molecules, and 2) there may be autoinducer molecules other than AI-2 (in the wild type strain) which are also involved in modulating expression of WrbA. The observation that not many proteins are regulated by AI-2 in E. coli O157:H7 raises the possibility that *E.coli* O157:H7 may be under the control of other autoinducer molecules. This is not surprising. Sperandio and co-workers have reported that another autoinducer molecule, AI-3 is presumably controlling some of the traits in *E. coli* O157:H7 (Sperandio *et al.*, 2003).

The key finding of this study was in *E. coli* O157:H7 only 3 proteins appear to be under the direct influence of the LuxS/AI-2 system. Nevertheless, the identified proteins are involved in carbohydrate, amino acid metabolism, and stress response. It would be interesting to identify whether the protein expression in *E. coli* O157:H7 would vary depending on the food matrix. There is a need for proteomic studies such as these to evaluate the interaction of food matrices and autoinducer molecules in controlling pathogen survival and virulence on foods.

CHAPTER V

PROTEOMIC ANALYSIS TO IDENTIFY THE ROLE OF AI-2 INHIBITORY FACTORS ON AI-2 INFLUENCED PROTEIN EXPRESSION OF *ESCHERICHIA COLI* O157:H7

OVERVIEW

Bacterial cell signaling utilizes autoinducer molecules for cell-cell communication in a process termed Quorum Sensing (QS). Several reports suggest that bacterial cells regulate different bacterial processes including virulence, bioluminescence, spoilage, and sporulation by utilizing autoinducer molecules for controlled gene and protein expression. In previous reports we have shown that autoinducer-2 (AI-2) molecules are involved in controlling different cellular processes in *E. coli* O157:H7. We have also previously shown that ground beef extracts possess compounds capable of inhibiting AI-2-controlled bioluminescence expression in *V. harveyi* and biofilm formation in *E. coli*. The objective of this work was to determine if inhibitory factors of AI-2 molecules present in ground beef extracts can also negate AI-2-influenced protein expression in *E. coli* O157:H7 cells. A two-dimensional gel electrophoresis-based proteomic approach was used in order to determine whether AI-2 inhibitory factors are capable of inhibiting AI-2-influenced protein expression in *E. coli* O157:H7 cells or not. The protein expression patterns between two experimental comparisons were studied namely, a) the *luxS* mutant and the *luxS* mutant supplemented with AI-2 molecules, and b) *luxS* mutant supplemented with AI-2 molecules and *luxS*

mutant supplemented with both AI-2 molecules and inhibitory factors. A total of 18 proteins were differentially expressed when in vitro-synthesized AI-2 molecules were added to the *luxS* mutant strain. When AI-2 inhibitory factors were added along with AI-2 molecules, the expression pattern of three AI-2-influenced proteins (GlmS, SpeE, and NikA) was changed. These results suggest that AI-2 molecules are involved in regulating cellular processes in *E. coli* O157:H7 and AI-2 inhibitors can negate the influence of AI-2 molecules on protein expression of selected proteins.

INTRODUCTION

Autoinducer (AI) molecules such as AI-1, AI-2, and AI-3 are thought to influence a variety of bacterial processes including pathogenicity, biofilms, motility and bioluminescence (Arevalo-Ferro *et al.*, 2003; Henke and Bassler, 2004; Pillai and Jesudhasan, 2007; Surette and Bassler, 1998). Optimal levels of these cell signaling (autoinducer) molecules and interaction of these molecules with regulatory proteins are key steps involved in the coordination of gene expression. Among these different autoinducer molecules, autoinducer-2 (AI-2) is synthesized by LuxS synthase, and the presence of the *luxS* gene has been identified in more than 55 species of Gram-negative and Gram-positive bacteria including *S*. Typhimurium (Xavier and Bassler, 2003).

It has been also shown that bacterial cells can produce autoinducer molecules when present in food matrices; however, the effect of these autoinducer molecules on bacterial behavior when present in food matrices is not well understood (Pillai and Jesudhasan, 2007; Smith *et al.*, 2004). Since foods are complex ecosystems, the fate and

behavior of microorganisms can be completely different in a particular food system. There are also reports suggesting that some food matrices also possess a characteristic ability to obstruct these autoinducer signaling molecules. Therefore, there is a growing interest in understanding how autoinducer molecules, pathogens and foods interact (Widmer *et al.*, 2007).

E. coli O157:H7 is a widespread human pathogen responsible for a high incidence of foodborne diseases. Most of the preventive measure to control this foodborne organism has focused on reducing viability; whereas relatively little effort of prevention has been based on understanding the molecular mechanisms of bacterial cell response when present in the food matrices. While genetic mechanisms for understanding behavior of microorganisms in food environment are not established, it is clear that microorganisms may develop resistance by adaptive response in a particular food environment (Berry, 2000). This increased adaptive response of organisms also enables them to resist inactivation processes or preservatives. In addition to developed resistance in the pathogen itself, this adaptive mechanism can even assist in better survival and virulence expression when present in the gastrointestinal tract (Mekalanos, 1992). Therefore, understanding the behavior of microorganisms in a food environment using a molecular approach can be significant for food safety and preservation.

Of the entire *E. coli* genome, about 5-10% of the genes are controlled by the AI-2 molecules (DeLisa *et al.*, 2001; Sperandio *et al.*, 2001). We have also previously shown that ground beef extracts contain compounds that are capable of inhibiting expression of AI-2 activity (Lu *et al.*, 2004). Understanding how these AI-2 molecules

and inhibitory factors interact with each other may give us insight into how certain foods may be susceptible to bacterial colonization or provide a unique means to control bacterial pathogens in certain foods. In the current study, Two Dimensional Gel Electrophoresis (2DGE)-Based Proteomic analysis couple with MALDI-MS was used to understand the effect of interaction between AI-2 molecules and inhibitory factors on protein expression of *E. coli* O157:H7.

MATERIAL AND METHODS

Preparation and quantification of AI-2 molecules. In vitro synthesized AI-2 was prepared and quantified as described previously (Sperandio *et al.*, 2003). Briefly, His-tagged LuxS and Pfs (proteins required for the formation of AI-2) were isolated and purified using a nickel resin column (Qiagen Inc., Valencia, CA). The purified enzymes were incubated with 1 mM S-adenosyl homocysteine (Sigma-Aldrich, St. Louis, MO) for one hour at 37° C, and AI-2 was further separated from the enzymes using a centrifuge column (Biomax-5, Millipore, Billerica, MA) and its concentration determined.

Preparation of ground beef extracts. To prepare ground beef extracts, ground beef patties were purchased from local market and mixed with deionized (DI) water (1:1 w/v) in stomacher. After stomaching, the supernatant was collected and filter sterilized using 0.2 um filter.

AI-2 bioassay to verify the inability of the luxS mutant to produce AI-2. Overnight cultures of *E. coli* O157:H7 VS 94 (*luxS* mutant) were inoculated (1:100) in fresh LB

broth (0.5% glucose) and cells were cultured at 37°C with shaking (100 rpm) and cell-free supernatants (CFS) were collected by centrifugation (10,000 x *g* for 2 min) at different time points. The supernatants were then passed through 0.22-µm syringe filters (Corning® Inc, Corning, NY) and stored at -20°C until used in AI-2 bioassay. *Vibrio harveyi* reporter strain BB170 (*lux*N::Tn5) (kindly provided by Dr. Bonni Bassler, Princeton University), which produces luminescence response in the presence of AI-2 molecules was used as a bioassay reporter strain to verify the lack of AI-2 production by the *luxS* mutant strain. AI-2 bioassay was performed as described earlier (Lu *et al.*, 2004). Briefly, 90 µl of the freshly diluted (1:5000) culture of the reporter strain in autoinducer bioassay (AB) medium were mixed with 10 µl of test samples (CFS) or 10 µl of AB media (negative control) in a 96-well plate. In addition the positive control (CFS from wild type *E. coli* O157:H7 (86-24)) was placed for each experiment. The plates were incubated at 30°C with moderate shaking (100 RPM) and the luminescence response of the reporter strains was monitored using a Wallac 1420 plate reader (PerkinElmer, Shelton, CT). Preparation of in vitro-synthesized AI-2 molecules was also checked using similar bioassay for their ability to induce luminescence response.

Inhibition assays of AI-2 activity. *V. harveyi* reporter strains BB170 was used to screen for AI-2 activity inhibition as described previously (Lu *et al.*, 2004). Briefly, 90 µl of the freshly diluted (1:5000) culture of the reporter strain in autoinducer bioassay (AB) medium were mixed with 5 µl of in vitro synthesized AI-2 and 5 µl of the sample (filter sterilized ground beef extracts) in a 96-well plate. Negative control (10 µl of AB media) and positive controls (5 µl of in vitro AI-2 + 5 µl of AB media) were included in each

experiment. Six replicates were used for each measurement. The microtiter plates were incubated at 30°C with moderate shaking (100 RPM) and the luminescence response of the reporter strains was monitored using a Wallac 1420 plate reader (PerkinElmer, Shelton, CT). Inhibition of AI-2 activity was expressed as a percentage relative to the corresponding positive control and was calculated as: 100 – [(light unit measurement of sample/light unit measurement of positive control) x 100].

Bacterial strain and growth conditions. An isogenic *luxS* mutant strain (VS 94) of wild type *E. coli* O157:H7 strain (86-24) (Kindly provided by V. Sperandio, Southwestern Medical Center, Dallas, TX) was used in this study. Overnight inoculum of bacterial cells were cultured at 37°C with moderate agitation (100 rpm) in LB medium, LB medium supplemented with 25 µM of AI-2 molecules, and LB medium supplemented with both 25 µM of AI-2 molecules and 20% (v/v) of ground beef extracts.

Protein sample preparation. *E. coli* O157:H7 cells were grown OD_{600} (~1.2) in the triplicate and bacterial cell pellets were collected by centrifugation (12000 × *g*). Collected cell pellets were washed three times using sterile DI water to remove any carryover from LB medium or beef extracts. Soluble protein fractions were extracted from each sample independently using B-Per® bacterial protein extraction reagent (Pierce, Rockford, IL). The Ready prep™ 2-D cleanup-kit (Bio-Rad, Hercules, CA) was used to reduce the ionic contaminants in the protein preparation. The purified proteins were dissolved in 100 µl of rehydration buffer [9.5 M urea, 2% w/v CHAPS, 18 mM DTT, 0.5% amphyolytes and one tablet of protease inhibitor (Roche Diagnostic,

Mannheim, Germany)] and insoluble proteins or cell debris were removed by centrifugation.

Two-dimensional gel electrophoresis. Protein concentrations were measured using the Bradford assay. Two hundred microliters (µl) of Bradford reagents were mixed with 1.7 µl of protein samples and absorbance was measured at 580 nm. Protein loads of 35 µg in 125 µl of rehydration buffer were used for 7 cm Immobilized pH Gradient (IPG) strips (pH 4-7). Rehydrations of IPG strips were conducted for overnight in rehydration tray (Bio-Rad, Hercules, CA). For the first dimensional electrophoresis, the isoelectric focusing of 7 cm IPG strips were conducted at a linear voltage gradient with ~15000 final voltage hours using Protean IEF cell (Biorad). After the required Vhs was applied, the IPG strips were incubated for 15 min in equilibration buffer I [6M urea, 30% glycerol, 2% SDS (w/v), 50 mM Tris- HCl (pH 8.8), 1% dithiotheritol and few grains of bromophenol blue] followed by 15 min in equilibration buffer II [6M urea, 30% glycerol, 2% SDS (w/v), 50 mM Tris- HCl (pH 8.8), 2.5 % iodoacetamide and bromophenol blue as color indicator]. Second dimension electrophoresis was performed at 150 constant volts using 10% SDS-PAGE gel. Protein spots were visualized using Sypro Ruby fluorescence stain (Molecular Probe, Eugene, OR) for the 7 cm IPG strip gel.

Data analysis. The extracted and purified proteins from three experimental replicates of each treatment (*E. coli* O157:H7 *luxS* mutant cells grown in LB medium, LB medium supplemented with 25 µM of AI-2 molecules, and LB medium supplemented with both 25 µM of AI-2 molecules and 20% (v/v) of ground beef extracts) were run in duplicate

using 7 cm IPG strips. Additional 2D gels were run from the 13 cm IPG strips to assist in spot excision. The gels were scanned using Gel Doc (Biorad, Hercules, CA) and the raw images were analyzed using advanced PDQuest 2-D gel analysis software version-8 (Bio-Rad, Hercules, CA). The protein expression patterns of; a) the *luxS* mutant and the *luxS* mutant supplemented with AI-2 molecules, and b) *luxS* mutant supplemented with AI-2 molecules and *luxS* mutant supplemented with both AI-2 molecules and inhibitory factors, were compared. Only those spots with spot intensities exhibiting -1.5 to + 1.5 fold change difference were short-listed for identification (Arevalo-Ferro *et al.*, 2005).

In-gel proteolytic digestion and Matrix Assisted Laser Desorption/Ionization- Mass Spectrometer (MALDI-MS). The desired protein spots of interest were manually excised (approximately 1 mm in size) and placed in a 96-well microtiter plate for their in-gel digestion. Proteolytic digestion was performed using trypsin 20 µg/mL (Promega, WI, USA) in 25 mM ammonium bicarbonate (ABC) and incubating at 37˚C overnight. The in-gel digested samples were than spotted onto MALDI targets using a ProMS™ (Genomic Solutions, Ann Arbor, MI) robot capable of sample clean-up prior to MS analysis. All MALDI-MS experiments were performed using a model 4700 Proteomics Analyzer MALDI-TOF/TOF (Applied Biosystems, Foster City, CA) instrument. The MS data for each gel spot was acquired using the reflectron detector and twenty tandem MS spectra per spot were acquired. All MS and MS/MS data were searched against the Swiss-Prot protein sequence database using the GPS Explorer (Applied Biosystems) software. The parameters for database searching were as follows: taxonomy, *Escherichia coli*; database, Swiss Prot; enzyme, trypsin; maximum missed cleavages, 1; variable

modifications, oxidation (Met); peptide tolerance, 85 ppm; and MS/MS fragment tolerance, 0.3 Da. To verify the reproducibility of MALDI-MS data, five spots were reanalyzed.

RESULTS

The AI-2 bioassay showed that luminescence by the CFS from *luxS* mutant remained at basal level (<100) during the growth period, confirming that the *luxS* mutant strain is unable to synthesize AI-2. In vitro-synthesized AI-2 molecules were able to induce luminescence (3.0×10^4 light units). Consistent with our previous report, the ground beef extracts inhibited the expression of AI-2 activity by more than 90% (Fig. 5.1).

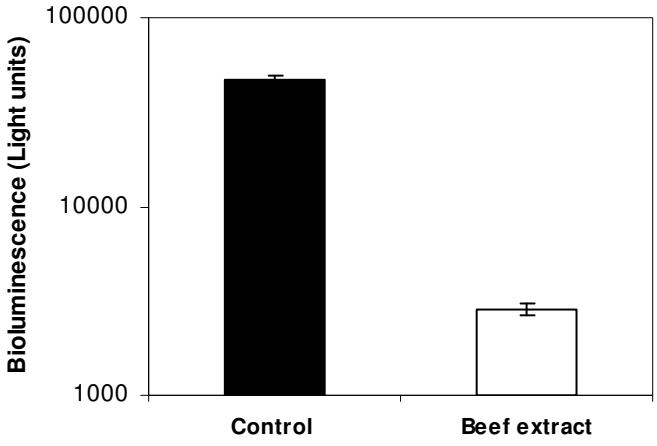


Fig. 5.1. Inhibition of AI-2-induced bioluminescence by ground beef extracts. The ability of ground beef extracts to inhibit bioluminescence expression was determined using the *V. harveyi* BB170 reporter strain based autoinducer bioassay.

We detected approximately 312 protein spots in three different experimental groups (*E. coli* O157:H7 *luxS* mutant cells grown in LB medium, LB medium supplemented with 25 µM of AI-2 molecules, and LB medium supplemented with both 25 µM of AI-2 molecules and 20% (v/v) of ground beef extracts). The measure of similarity in spotting patterns between individual gels resulted in correlation coefficients in the range of 0.66 to 0.99, implying strong correlation of the protein spotting patterns among the different gels (Bland et al., 2006). When analyzed, eighteen protein spots were found to be differentially expressed (4 up-regulated and 14 down-regulated) when *luxS* mutant was supplemented with AI-2 (Table 5.1) and nine protein spots were found to be differentially expressed (2 up-regulated and 7 down-regulated) when *luxS* mutant strain was grown in the presence of AI-2 and inhibitory factors (Table 5.2). The protein spots were identified using MALDI-MS and resulted in protein hits with confidence intervals of 95% and 100%. The expression pattern of three AI-2-influenced proteins (GlmS, SpeE, and NikA) was changed when AI-2 inhibitory factors were added along with AI-2 molecules (Table 5.3). However, in the presence of these inhibitory factors, the expression pattern of remaining fifteen AI-2 influence proteins (DsbC, YbbN, UspE, Tsx, GshB, SseA, PfkA, Eda, Eno, GpmA, IscC, FruB, SspB, YnjE, WrbA) did not change.

Table 5.1. Differentially expressed proteins of *E. coli* O157:H7 *luxS* mutant in the presence of AI-2.

ID	Protein Identification	Gene	*Fold Change (AI-2)	Functions
1	Thiol: disulphide interchange protein dsbC	*dsbC*	-2.8	Posttranslational modification, protein turnover, chaperone
2	Protein ybbN	*ybbN*	-1.7	Posttranslational modification, protein turnover, chaperone
3	Universal stress protein E	*uspE*	-2.2	Oxidative stress resistance
4	Nucleoside specific channel forming protein	*tsx*	-3	Cell wall/ envelop biogenesis/ membrane
5	Glucosamine- fructose- 6 phosphate aminotransferase	*glmS*	3	Cell wall/ envelop biogenesis/ membrane
6	Sperimidine synthetase	*speE*	-2.7	Amino acid transport and metabolism

Table 5.1 Continued

ID	Protein Identification	Gene	*Fold Change (AI-2)	Functions
7	Gluthathione synthetase	*gshB*	-2.3	Coenzyme transport and metabolism
8	3-mercaptopyruvate sulphur transferase	*sseA*	-2.2	Inorganic ion transport and metabolism
9	6-phosphofructokinase isozyme I	*pfkA*	7.3	Carbohydrate transport and metabolism
10	4- hydroxy-2-oxoglutarate adolase	*eda*	-2.2	Carbohydrate transport and metabolism
11	Nickel binding periplasmic protein binding precursor	*nikA*	-2.4	Amino acid transport and metabolism
12	Enolase	*eno*	2.1	Carbohydrate transport and metabolism
13	2,3 biphosphoglycerate dependent phosphoglycerate mutase	*gpmA*	1.8	Carbohydrate transport and metabolism

Table 5.1 Continued

ID	Protein Identification	Gene	*Fold Change (AI-2)	Functions
14	Cystein desulfurase	*iscC*	-3	Amino acid transport and metabolism
15	Fructose specific IIA/FPr component (EIIA-Fru)	*fruB*	-2.6	Carbohydrate transport and metabolism
16	Stringent starvation protein	*sspB*	-3.2	Stress response
17	Putative thiosulphate sulfurtransferase ynjE precursor	*ynjE*	-2.6	Putative protein
18	Trp repressor binding protein	*wrbA*	-1.8	Repressor binding protein

* Fold difference in the protein expression of *luxS* mutant strain compared to *luxS* mutant strain grown in the presence of 25 µM of AI-2

Table 5.2. Differentially expressed proteins of *E. coli* O157:H7 *luxS* mutant in the presence of AI-2 and inhibitors.

ID	Protein Identification	gene	*Fold Change (AI-2 + inhibitors)	Functions
1	Putrescine-binding periplasmic protein precurssoe (SPBP)	*potD*	-2.2	ABC transporters, Metabolisms
2	Chaperone protein dnaK(heat shock protein)	*dnaK*	-2.12	Cellular Processes and Signaling, Post translational modifications
3	3-oxoacyl- (acyl carrier protein) synthase III	*fabH*	-5.9	Fatty acid biosynthesis
4	Spermidine synthase	*speE*	2	Amino acid transport and metabolism
5	Seryl-tRNA synthetase	*serS*	-2.9	Information Storage and Processing

Table 5.2 Continued

ID	Protein Identification	gene	*Fold Change (AI-2 + inhibitors)	Functions
6	Malate dehydrogenase	*Mdh*	-9.1	Energy production and conversion, Metabolisms
7	Nickel-binding periplasmic protein precursor	*nikA*	2.1	ABC transporter
8	Glucosamine-fructose-6-phosphate aminotransferase	*glmS*	-5.6	Cell wall/membrane/envelope biogenesis, Cellular Processes and Signaling
9	Periplasmic oligopeptide binding protein	*oppA*	-1.8	Oligopeptide transport; periplasmic binding protein, Metabolisms

*Fold change calculation based on the difference of the mean spot intensity between combined AI-2 and inhibitors to AI-2 treatment.

Table 5.3. Proteins that were differentially expressed in the presence of AI-2 and modulated when inhibitory factors were added along with AI-2 molecules.

ID	Protein Identification	Gene	AI-2	AI-2 + Inhibitors
1	Glucosamine- fructose- 6 phosphate aminotransferase	*glmS*	3	-5.6
2	Sperimidine synthetase	*speE*	-2.7	2
3	Nickel binding periplasmic protein binding precursor	*nikA*	-2.4	2.1

DISCUSSION

The potential role of autoinducer molecules in modulating microbial persistence, growth and virulence traits is intriguing. Our understanding of microbial cell signaling molecules, signaling pathways, and how these processes control microbial growth and virulence traits in foods is still very much in its infancy (Pillai and Jesudhasan, 2007; Smith *et al.*, 2004). There are reports that certain food products contain AI-2 molecules and some can even exhibit AI-2 inhibitory activity (Bjarnsholt *et al.*, 2005; Lu *et al.*, 2004, 2005). The source of AI-2 could be microbial in origin or foods could contain compounds that can mimic AI-2 autoinducer activity (Teplitski *et al.*, 2004). Lu and others (2004) have reported that many meat products including ground beef meat are

capable of inhibiting AI-2 activity when assayed using *V. harveyi* reporter strain (Lu *et al.*, 2004). Consistent with earlier report, the ground beef extracts used in this study were able to inhibit the expression of AI-2 activity in the classical AI-2 sensing *V. harveyi* biosensor assays.

Proteins associated with stress response, carbohydrate metabolism, amino acid metabolism were modulated by the presence of AI-2 molecules (Table 5.1). These results suggested that AI-2 may have a pivotal role in regulating different cellular processes in *E. coli* O157:H7 cells. In this study, a total of nine proteins were identified as differentially expressed in the presence of AI-2 inhibitory factors present in ground beef extracts. Key proteins modulated by inhibitory factors were related to the ABC transporter system (PotD, NikA), metabolism (FabH, SpeE, Mdh, OppA, GlmS), information storage and processing (SerS), and protein related to heat shock (DnaK). The heat shock proteins (Hsp's) are important in the bacterial stress response. Many of the heat shock proteins are molecular chaperones that bind to nascent, misfolded, or damaged polypeptides and assist them in reaching a native conformation. Chaperones of *E. coli* include Hsp60 (GroEL), Hsp70 (DnaK), Hsp100, and the small heat shock proteins IbpA and IbpB. The latter category is believed to assist the refolding of denatured proteins in the presence of other chaperones (Malone *et al.*, 2006).

Our study suggests that AI-2 modulates protein expression in *E. coli* O157:H7 and the interaction of the AI-2 molecules with inhibitors of ground beef extracts also have some influences on the protein expression. A total of three proteins (GlmS, SpeE, and NikA) were found to be common when the response from two treatments (AI-2 or AI-2

with inhibitory factors) was compared. These result suggested that although AI-2 inhibitory factors are not able to modulate the expression of all AI-2 influence proteins, it atleast modulates the expression of selected AI-2 regulated proteins. Overall, these results suggest that protein expression in *E. coli* O157:H7 can be modulated depending on whether cells are in contact with AI-2 molecules in the presence or absence of ground beef derived inhibitory factors.

CHAPTER VI

SUMMARY

Cell-cell communications in bacterial cells is thought to be mediated by small diffusible hormone like molecules in a process termed Quorum Sensing. Signaling molecules produced by bacterial cells influence variety of cellular processes including virulence. Therefore, there is a continuing interest in identifying compounds that can interfere with the ability of bacterial cells to respond to cell signaling molecules. One class of signaling molecules, AI-2, produced by a large number of bacteria including *E. coli* is considered to be very important, since AI-2 molecules are used for inter-species communication. Previous work in our laboratory demonstrated that ground beef contains compounds that interfere with AI-2 mediated bioluminescence expression in *V. harveyi*. In this context further studies were conducted to isolate and characterize the AI-2 inhibitory factors present in ground beef and to determine how inhibitory factors modulate the AI-2 responses in *E. coli* O157:H7 cells.

IDENTIFICATION AND CHARACTERIZATION OF AI-2 INHIBITORS PRESENT IN GROUND BEEF EXTRACTS

Using a solvent extraction procedure and gas chromatography analysis the AI-2 inhibitory factors present in ground beef extracts were identified as both medium and long chain fatty acids. When selected fatty acids such as palmitic acid (C 16:0), stearic acid (C 18:0), oleic acid (C 18:1 ω9), and linoleic acid (C 18:2 ω6) were tested for AI-2

inhibition at different concentrations (1mM, 5mM, and 10mM), AI-2 inhibition ranging from 25% to 90% was observed. A combination of these selected fatty acids at a concentration equivalent to that observed in GC analysis also inhibited the expression of AI-2 induced bioluminescence by 52-65%. Both ground beef extracts and the combined mixture of selected fatty acids also resulted in reduced AI-2-influenced biofilm formation by *E. coli* K12 cells. Our results demonstrate that both medium and long chain fatty acids in ground beef have the ability to interfere with AI-2-based cell signaling.

IDENTIFICATION OF LUXS/AI-2 MEDIATED PROTEIN EXPRESSION IN *ESCHERICHIA COLI* O157:H7 USING TWO DIMENSIONAL GEL ELECTROPHORESIS

The role of *luxS*/AI-2 on the *E. coli* O157:H7 cellular protein expression was studied using a 2D gel electrophoresis-based proteomic approach. When the protein expression patterns between a wild type *E. coli* O157:H7 and its isogenic *luxS* mutant was compared, total of eleven proteins were identified to be differentially expressed. In the presence of in vitro-synthesized AI-2 molecules eighteen proteins were differentially expressed in the *luxS* mutant strain of *E. coli* O157:H7. Three proteins (WrbA, GpmA, and YbbN) were common between two experimental comparisons (wild type vs *luxS* mutant and *luxS* mutants vs *luxS* mutant strain supplemented with AI-2) suggesting the definite role of AI-2 molecules produced by *luxS* gene in modulating protein expression of these three proteins. The FliC protein, which is involved in flagellar synthesis and motility, was up-regulated in the wild type strain, but was not influenced by the addition

of synthetic AI-2 molecules to the *luxS* mutant suggesting the involvement of signaling molecules other than AI-2 on flagellar synthesis and motility.

THE ROLE OF AI-2 INHIBITORY FACTORS ON AI-2 INFLUENCED PROTEIN EXPRESSION OF *ESCHERICHIA COLI* O157:H7

In a previous report we have demonstrated that autoinducer-2 (AI-2) molecules are involved in controlling different cellular processes in *E. coli* O157:H7. We also previously showed that ground beef extracts possess compounds capable of inhibiting AI-2-controlled bioluminescence expression in *V. harveyi* and biofilm formation in *E. coli*. The objective of this work was to determine using protein analysis if inhibitory factors of AI-2 molecules present in ground beef extracts can also negate AI-2-influenced protein expression in *E. coli* O157:H7 cells. The protein expression patterns between the *luxS* mutant, the *luxS* mutant supplemented with AI-2 molecules, and *luxS* mutant supplemented with both AI-2 molecules and inhibitory factors were studied. A total of 18 proteins were differentially expressed when in vitro-synthesized AI-2 molecules were added to *luxS* mutant strain. When AI-2 inhibitory factors were added along with AI-2 molecules, the expression patterns of three AI-2-influenced proteins (GlmS, SpeE, and NikA) were changed. These results suggest that AI-2 inhibitors can negate the influence of AI-2 molecules on protein expression of selected proteins.

CHAPTER VII

CONCLUSIONS

1. Ground beef contains medium chain and long chain fatty acids that are able to inhibit AI-2-controlled bioluminescence expression in *V. harveyi* and AI-2-influenced biofilm formation in *E. coli* cells.
2. Protein expression analysis using two dimensional gel electrophoresis shows that LuxS/AI-2 system modulates protein expression in *E. coli* O157:H7. Differentially expressed proteins are involved in carbohydrate and amino acid metabolism, stress response, and flagella and motility genes. These results suggest that LuxS/AI-2 system has a critical role in modulating different cellular processes in *E. coli* O157:H7.
3. Interaction of the AI-2 molecules with inhibitors of ground beef extracts modulates protein expression in *E. coli* O157:H7 cells and the extent of this effect depend on whether cells are in contact with AI-2 molecules in the presence or absence of ground beef-derived inhibitory factors. The effects of AI-2 molecules on expression of three proteins (GlmS, SpeE, and NikA) were negated in the presence of AI-2 inhibitory factors suggesting that AI-2 inhibitory factors modulate the expression of selected AI-2 regulated proteins.

CHAPTER VIII

FUTURE RESEARCH DIRECTION

1. Three different polarity solvents (hexane, ethyl acetate, and methyl ether ketone) were used for extracting inhibitory factors present in ground beef. Although hexane extract showed highest level of AI-2 inhibition (~90%), both ethyl acetate and methyl ether ketone also showed some level (~20-25%) of AI-2 inhibition. Identification of inhibitory factors present in ethyl acetate and methyl ether ketone extracts should be performed in order to fully characterize all the inhibitory factors present in ground beef extracts.
2. We identified that selected fatty acids have the ability to inhibit AI-2-influenced bioluminescence and further experimentation is necessary to determine the mechanism by which these fatty acids-based inhibitors inhibits the bioluminescence production in the *V. harveyi* reporter strain. Also, one of the major draw backs in utilizing this assay is that it is not possible to determine if the observed inhibition is simple interference with bioluminescence. To rule out possible interference with bioluminescence as such, reporter strain should be constructed where bioluminescence is constitutive and independent of quorum sensing phenomenon.
3. Based on the preliminary experiments using varying gradient immobilized pH strips (IPG) we observed that most of the soluble proteins were falling under the pH range of 4-7. To obtain a complete protein profile of soluble protein the

varying pH IPG strips that can detect maximum protein spots should be used. The length of IPG strips used in these studies was 7 cm, therefore, two or more protein might appear as a single protein spot if their isolectric *pI* is very close. It would be advantageous to use bigger length IPG strips (11 cm or 13 cm), which can result in better separation of proteins.

4. Identifying the role of LuxS/AI-2 system in *E. coli* O157:H7 focused on the soluble protein fraction and, therefore, the possibility that LuxS/AI-2 also controls the expression of proteins present in insoluble and secreted proteins can not be excluded. Both insoluble and secreted protein should be used to investigate the complete effect of LuxS/AI-2 on *E. coli* O157:H7 protein expression.
5. In protein expression analysis we used 25 μM of AI-2 concentrations in experiments designed to supplement *luxS* mutant strain of *E. coli* O157:H7 with exogenous AI-2 molecules. Different concentrations of AI-2 molecules should be used to investigate the effect of varying concentration of AI-2 on protein expression.
6. Production or utilization of AI-2 molecules by *E. coli* cells is tested using *V. harveyi* reporter strain although both *E. coli* and *V. harveyi* cells have different receptor proteins (LuxP in *V. harveyi* and Lsr operon in *E. coli*) for responding to AI-2 molecules. It would be more appropriate to construct and use *E. coli* reporter strain where AI-2 response can be quantified by utilizing *E. coli* strain rather than relying on *V. harveyi* reporter strain.

7. Most of the studies that are performed to understand the role of AI-2 molecules in bacterial cell response are in vitro and laboratory-based. However, in reality (in vivo) there could be a number of factors that could determine the outcome of bacterial response to cell signaling. In the future, in vivo experiments should be performed to precisely understand the effect of AI-2 molecules.

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APPENDIX A

PROTEIN ANALYSIS USING TWO DIMENSIONAL GEL ELECTROPHORESIS

Extraction of soluble protein fraction from *E. coli* O157:H7 cells.

- Take 1.5 ml of logarithmic phase grown culture of *E. coli* cells in micro centrifuge tube.
- Centrifuge the tube at maximum rpm for 3 min.
- Discard the supernatant carefully
- Again add 1.5 ml of logarithmic phase grown culture of *E. coli* cells in the same centrifuge tubes
- Centrifuge the tube at maximum rpm for 3 min.
- Discard the supernatant carefully.
- To ensure that supernatant is completely removed, re centrifuge the pellet for 1 min and remove leftover supernatant
- Total volume of culture centrifuges for one tube will be 3.0 ml

Cell lysis using B-per cell lysis buffer.

- Add 300 microliter of B-per cell lysis buffer in the micro-centrifuge tube containing cell pellet.
- Vortex the content vigorously for 5 min.
- Centrifuge the tube at maximum rpm for 3 min

- Collect the supernatant and place into new micro-centrifuge tube. This content represents soluble protein fraction.
- Discard the pellet

Protein clean-up using ReadyPrep 2-D cleanup kit. The purpose of using commercially available ReadyPrep 2-D cleanup kit was to enhance the suitability of prepared protein samples for 2DGE by reducing the ionic contamination. Moreover, this procedure also results in concentration of diluted samples thereby allowing higher protein loads during gel electrophoresis. The detailed procedure used in the sample clean-up is as follow.

- Three hundred microliter of prepared soluble protein extracts were mixed with 300 µl precipitating agent 1 in eppendorf tube followed by incubation on ice for 15 min.
- At the end of incubation period, 300 µl precipitating agent 2 was added to the mixture of protein and precipitating agent 1 followed by proper mixing using vortexer and centrifugation of the eppendorf tubes at maximum speed for 5 min. The supernatants were removed immediately and tubes were placed again for 1 min centrifugation. At the end of second centrifugation any remaining supernatants were removed.
- At this time point 40 µl of wash reagent 1 on top of the pellet and tubes were centrifuge at maximum speed (> 12,000 x g) for 5 min followed by discarding supernatant. After removing supernatant, 25 µl of ultrapure water was added on

the top of the pellet and tubes were vortexed for 30 sec (prepared protein pellet does not dissolve in water).

- Protein pellets were further treated by adding 1 ml of prechilled (20°C) wash reagent 2, 5 µl of wash 2 additive, and tubes were vortexed for 1 min. The tubes were finally incubated at -20°C for 30 min with intermediate vortexing at every 10 min. After the incubation period, tubes were centrifuged at maximum speed for 5 min and supernatant was discarded. Tubes were placed again for 1 min centrifugation to discard any remaining supernatants and formed protein pellets were air dried for 5 min.

Resuspending protein pellet in sample/rehydration buffer.

- The protein pellets achieved after step 3 were used in this step.
- Take 100 microliter of sample/ rehydration buffer and re-suspend protein pellet into it.
- Vortex very very vigorously for complete solubalization.
- Centrifuge the content at maximum speed for 1 min.
- Takeout the supernatant portion in fresh tube and discard the tube containing cell debris.
- The supernatant portion is ready to use for Bradford bioassay

Bradford Bioassay.

Bradford bioassay for the protein quantification was performed in a 96-well microtiter plate. The bradford assay is a colorimetric assay in which acidic solution of bradford dye reagent (Coomassie) shifts from 465 nm to 595 nm when binding with

protein occurs. Increased absorbance at 595 nm is proportional to the amount of bound dye and thereby to the amount of protein present in the sample. The detailed procedure of the bradford bioassay for the protein quantification is described below.

In order to determine the protein concentration of unknown samples, standard protein curve was prepared using known protein concentration. Protein standards to prepare a standard curve were prepared from known concentration of bovine serum albumin (BSA). Protein standards were prepared by dissolving different concentrations of BSA (0 to 2000 µg/mL) in deionized water. Five microliter of this prepared protein standards were mixed with 200 µl of the bradford dye reagent and absorbance reading (590 nm) were reported. Obtained absorbance reading were plotted against concentration of the protein standards using excel spread sheet and equation of line was derived. To measure the concentration of unknown protein samples, known volume of protein sample was mixed with 200 µl of the bradford dye reagent and absorbances reading were obtained. Later the protein concentrations from the unknown protein samples were determined by solving the line equation using A590nm of the unknown protein samples.

Rehydration of the strips.

- Take 35 µg of protein load (based on Bradford assay concentration determination) and male the total volume of 125 µl using rehydration buffer (note, above mentioned concentration of 35 µg protein is for 7 cm IPG strips. When bigger gels are run for spot excision purpose the protein load should be ~500 µg.

- Carefully overlay prepared 125 µl content in rehydration tray and put the IPG strips carefully on this content. Overlay 1 ml of mineral oil to prevent drying out of strips.

Step 7. Focusing on IPG strips in first dimension.

- Remove the rehydrated strips from rehydration tray and remove mineral oil by vertically tapping on soft paper.
- Place small pieces of filter paper on focusing tray electrodes to avoid burning of IPG strips during 1st dimension voltage application.
- Place strips on focusing tray, add 1 ml of mineral oil on strip and perform 1st dimensional separation for desired voltage (S1: 150 V-hr in 15 min, S2: 4000 V-hr in 2 hrs, S3: 10000 V-hr for 2.5 hr, and holding at 500 V-hr).
- Take out the IPG strips and try to remove mineral oil by vertically tapping on tissue paper).

Equilibration of IPG strips.

- Add 100 mg of DTT in the 1st equilibration buffer tube (10 ml content) and dissolve it completely
- Place the IPG strips in tube and shake it for 15 min on shaker.
- Add 250 mg of iodoacetamide in the 2nd equilibration buffer tube (10 ml content) and dissolve it completely
- Take the IPG strips from 1st tube and place it on 2nd tube. Shake it for another 15 minutes.

SDS-PAGE for 2nd dimensional separation.

- Use 10% SDS-PAGE gel to cast in 1mm spacer glass assembly (should not have comb). Add methanol on the top of gel layer (immediately after pouring). Addition of methanol helps to get straight top layer of gel.
- Insert the IPG strip carefully (should touch perfectly to the top layer of SDS-PAGE gel) and run for the second dimension on SDS- PAGE
- Condition for the SDS-PAGE: at 150 volts for 1 hr.

Staining.

- After running the SDS-PAGE, take out the gels in clean tray and wash it with water for 3 times (5 minutes each).
- Replace the water with fixing solution and fix the gel for 15 min.
- Add staining reagent (Sypro Ruby) and keep on shaker for overnight.
- Remove the staining reagent with washing solution and keep on shaker for 15 min.
- Gels are ready for imaging.

Recipes

Sample/rehydration buffer.

9.5 M urea	5.7 gm
2% CHAPS	200 mg
30 mM DTT	0.05 gm
0.5% ampholytes	50 microliter

1 tablet of protease inhibitor

Few grains of bromophenol blue

Make volume up to 10 ml and dissolve completely

Equilibration buffer.

50 mM Tris- HCL, pH 8.8	6.7 ml of 1.5M
6 M urea	72 gm
2% SDS	4 gm

Few grains of bromophenol blue

Make volume up to 200 ml and dissolve completely

Running buffer.

25 mM Tris-base	3 gm
192 mM glycines	14.4 gm
0.1% SDS	1 gm

Make volume up to 1 lit and dissolve completely

Fixing solution.

50% methanol	500 ml
7% glacial acetic acid	70

Make volume up to 1 lit using DI water.

Washing solution.

10% methanol	100 ml
7% glacial acetic acid	70 ml

Make volume up to 1 lit using DI water.

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